REGULATION OF THE P38 MAPK SIGNALING PATHWAY BY THE CIRCADIAN CLOCK

A Dissertation

by

CHARLES SIDNEY GOLDSMITH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Deborah Bell-Pedersen
Committee Members,	Paul E. Hardin
	Bruce B. Riley
	Heather H. Wilkinson
Intercollegiate Faculty Chair,	Craig J. Coates

August 2013

Major Subject: Genetics

ABSTRACT

Mitogen activated protein kinase (MAPK) pathways are conserved biochemical signal transduction pathways in eukaryotic organisms. These signaling pathways demonstrate great versatility in their ability to detect various environmental stimuli and direct an appropriate cellular response. The circadian clock is a timekeeping mechanism that temporally coordinates diverse biological functions in an organism with the environment. Thus, it is not surprising that MAPK pathways have been utilized by the circadian clock to regulate many essential functions. Due to the conserved nature of circadian clocks and MAPK signaling pathways in eukaryotes, it is possible to develop hypotheses in simple model organisms, such as the fungus Neurospora, that are relevant to more complex organisms.

The OS-2 MAPK pathway in the filamentous fungus Neurospora is rhythmically activated by the circadian clock. In order to generate this rhythmic signal, the circadian oscillator directly regulates the rhythmic transcription of the *os-4* MAPKKK and histidine phosphotransferase *hpt-1*, which are upstream regulators of the OS-2 MAPK. Also, the circadian rhythm in MAPK activation produces a more robust stress response during the time of the day that stress is most likely to be encountered. Based on these data, a model for the clock regulation of MAPK activation is presented, and a biological significance is assigned to the rhythms in this pathway.

Informed by these findings in Neurospora, the related p38 MAPK pathway was studied in mammalian cell lines that represent functionally distinct tissues in regards to clock function. A rhythm in p38 MAPK activation was observed in cells derived from the suprachiasmatic nucleus and fibroblasts of a mouse, the master pacemaker and a peripheral tissue, respectively. In cells that lacked a functional circadian oscillator, the rhythm in p38 activation was absent, and overall levels of p38 protein were lower. These data demonstrate a circadian clock-dependent oscillation in p38 activity.

These studies provide a basis to understand how the circadian clock generates endogenous rhythms in MAPK signal transduction pathways. Also, the characterization of clock-regulated stress response pathways provides an understanding of the adaptive advantage of the circadian clock.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

CIRCADIAN CLOCKS

Life on our planet is constantly bombarded by a myriad of stressful conditions. In order to survive, the cell has developed mechanisms to aid in adapting to these stressors (Dunlap et al., 2004). Signal transduction pathways provide a biochemical means for the cell to sense that the cell is under stress and relay a signal that will lead to an appropriate response. This response will typically help the organism in the short term, in the span of minutes to hours. However, certain stressors mediated by the rotation of the earth such as sunlight, or the daily increase in temperature, provide a cyclic stress to organisms (Dunlap et al., 2004). Without a means to anticipate these predictable daily events, the organism would be playing a constant game of catch up to mount an appropriate response. Alternatively, the organism would waste precious resources by providing protection during times when it is not needed, particularly during the night (Dunlap et al., 2004). It makes sense, then, that organisms have evolved a means to tell time. The circadian clock is a biological mechanism that has bestowed a time-sensing ability to organisms as simple as cyanobacteria and fungi, or as complex as humans (Bell-Pedersen et al., 2005). Upon gaining a sense of time, organisms are able to anticipate

daily stresses such as the ultraviolet (UV) radiation from the sun (Vitalini et al., 2007), and also, can coordinate certain aspects of their biology to a time of day that is most advantageous. For example, fungi produce asexual spores when the sunlight is absent and the humidity is higher (Dunlap et al., 2004), and cyanobacteria are able to temporally segregate two incompatible, but essential biological functions: photosynthesis and nitrogen fixation (Berman-Frank et al., 2003). Another consequence of biological timekeeping is that organisms can occupy temporal niches (Dunlap et al., 2004). Complex organisms can avoid certain predators during the day, and evolve characteristics that are suitable for their temporal niche such as echo-location in bats or camouflage for daytime animals (DeCoursey and Krulas, 1998; Dunlap et al., 2004).

ORGANIZATION OF THE CIRCADIAN CLOCK

The proper functioning of a circadian clock requires the integration of many biological processes (Figure 1-1). At the core of a circadian clock, a molecular oscillator cycles with a period of around 24 hours and acts as a pacemaker to generate endogenous rhythms to match the daily solar cycle (Dunlap et al., 2004). It appears that organisms have evolved these oscillators independently, as the genes that comprise oscillators are not conserved across phyla (Tauber et al., 2004) (Figure 1-2). Although, in animals, oscillator components are related by descent (Tauber et al., 2004) (Figure 1-2). Despite the dissimilarity of oscillator genes across phyla, the oscillators share a common organizational principle: they function as a negative feedback loops (Cahill, 2002;

Dunlap et al., 1999; Hardin, 2005; Ko and Takahashi, 2006) (Figure1-1). Conceptually, a positive element(s), usually a transcriptional activator, induces the expression of the negative element(s). The negative element(s), upon sufficient accumulation, begins to repress the activity of positive element(s). The negative element(s) endure persistent phosphorylation, which over time, lead to their degradation. As the repression mediated by the negative components is diminished through degradation, the positive element(s) can once again function as a transcriptional activator, and the cycle restarts the next day (Dunlap, 1999).

The ability to tell time would be useless if internal time was not in synchrony with the environment, or if cells within a tissue were not synchronized to each other. Therefore, input pathways to the circadian oscillator are vital to maintaining the proper synchrony of the oscillator. In a process called entrainment, input pathways reset the oscillator so that the period of the oscillator conforms to the 24h period of the environment (Johnson et al., 2003) (Figure 1-1). To reset the oscillator, input pathways utilize various mechanisms, but in general, they use a mechanism that either increases or decreases the levels or activity of a component of the molecular oscillator (Dunlap, 1999). In this way, input pathways detect temporal cues and impinge on the oscillator so that the clock can be set to the correct time. One of the most ubiquitous time giving cues, or zeitgebers, is light, but non-photic environmental cues including temperature, nutrition, and social interactions can also entrain the circadian clock (Dunlap, 1999; Johnson et al., 2003; Mohawk et al., 2012). In addition, the clock can utilize a strategy, called gating, that

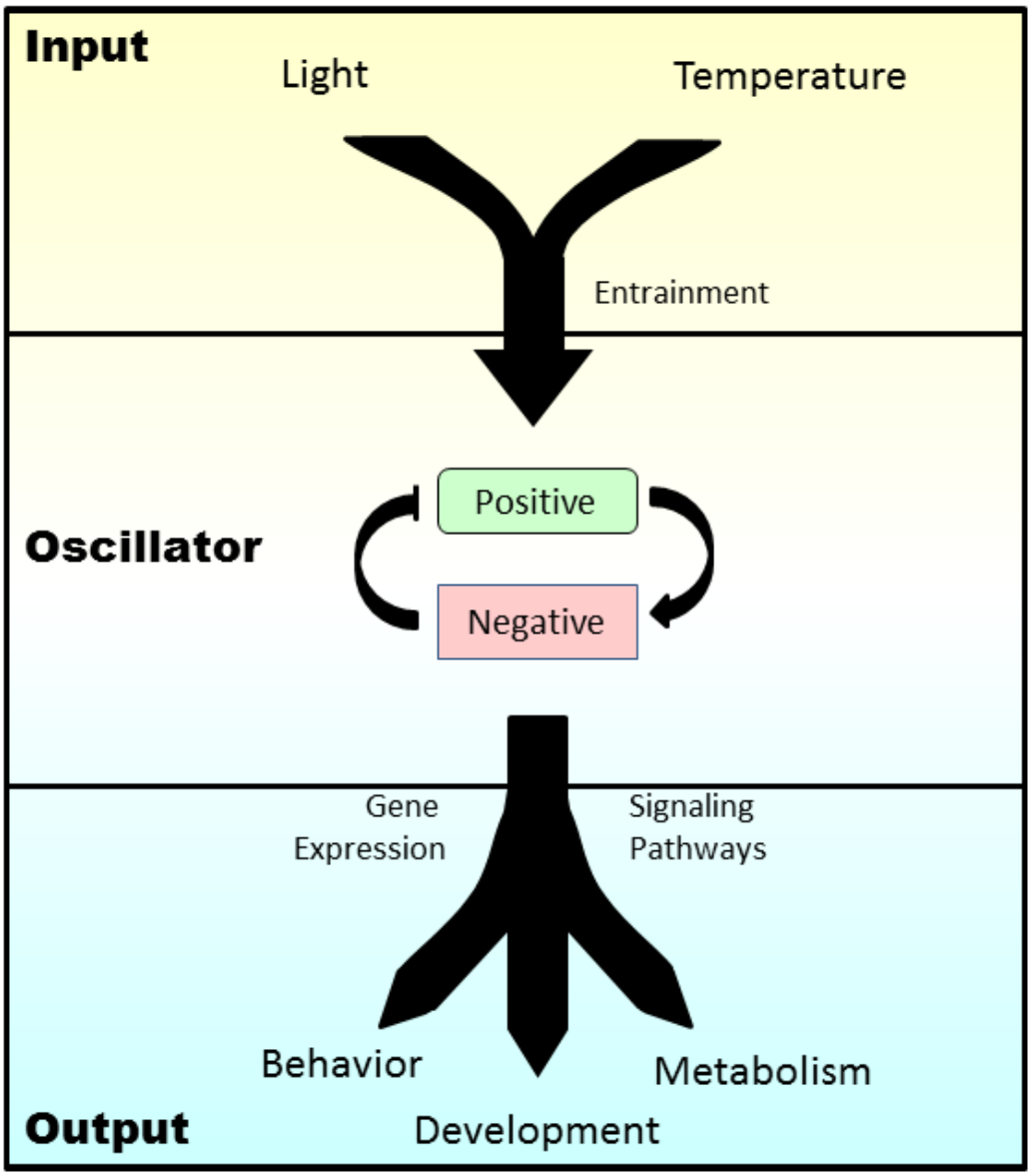


Figure 1-1. Organization of the circadian clock. The core of a circadian clock is its timekeeping molecular oscillator that cycles with a period of approximately 24 hours. Input pathways detect temporal cues in the environment, such as light and temperature, and synchronize the molecular oscillator to external time through a process called entrainment. Finally, output pathways couple the molecular oscillator to the control of gene expression and signaling pathways. Rhythmic control of output pathways by the clock underlies overt circadian rhythms in behavior, metabolism, and development.

	Mouse Rat	Chicken	Zebrafish	Neurospora	Drosophila
Positive	Clock Bmal1	Clock Bmal1	Clock Bmal1	WC-1 WC-2 (WCC)	Clock Cycle
Negative	Per1 Cry1 Per2 Cry2 Per3 Cry3	Per1 Cry1 Per2 Cry2 Per3 Cry3	Per1 Cry1 Per2 Cry2 Per3 Cry3	FRQ FRH	Per Tim

Figure 1-2. Molecular components of the circadian oscillator in diverse eukaryotic organisms. Components of the molecular oscillator are categorized by their involvement in the positive or negative arm of the oscillator. Clock genes in animals show strong sequence conservation. Conversely, comparison of clock genes between phyla reveals that clock genes are not related by descent.

restricts the perception of some environmental cues at certain times of day. For example, most mammals are insensitive to a light pulse during the day, but during the night, a light pulse can reset the clock (Daan and Pittendrigh, 1976). Through the use of gating, the circadian clock regulates the time of day when the clock can be reset, and, therefore, the clock can successfully entrain to the environment (Heintzen et al., 2001).

In organisms of different complexity, cells vary in their ability to support a functional oscillator. In unicellular organisms, each cell has a fully entrainable oscillator that predominantly responds to light (Bell-Pedersen et al., 2005). However, in complex multicellular organisms, not all cell types have the necessary sensory capabilities, such as photoperception, to entrain their circadian oscillator (Mohawk et al., 2012). The cellular oscillators and overall rhythmicity of the organism are broken down into the components of the central pacemaker and peripheral oscillators (Mohawk et al., 2012). Organisms possessing a nervous system typically delegate the ability to sense environmental cues to the nervous system rather than to individual cells in order to integrate multiple sensory inputs (Mohawk et al., 2012). In general, sensory inputs are integrated in the brain where special oscillating cells, referred to as the master pacemaker, entrain the oscillators of all other tissues in the organism (Mohawk et al., 2012). The relationship between the master pacemaker and peripheral oscillators varies between species. For instance, in Drosophila, dissociated body parts have functional circadian oscillators, and are able to entrain to light:dark (LD) cycles (Plautz et al., 1997). Despite the ability of peripheral oscillators to independently entrain, Drosophila

still possesses a neuronal mechanism to entrain pacemaker neurons in the brain, which are important for rhythms in complex behavior, such as locomotion and eclosion (Hardin, 2005). In mammals, light is perceived by non-visual retinal ganglia that transmit information via neural connections to a region of the hypothalamus called the suprachiasmatic nucleus (SCN) (Dibner et al., 2010). The SCN synchronizes oscillators in other tissues by a mechanism that utilizes input pathways in individual cells to reset the peripheral oscillator (Dibner et al., 2010). In addition to maintaining entrainment of peripheral oscillators with the environment, this mechanism also ensures that cellular oscillations within tissues are properly in phase so as to provide resonance between individual cellular rhythms. In summary, functional oscillators in individual cells require the intrinsic ability to be reset by input pathways, even though the perception of environmental cues may be delegated to other parts of the organism.

Another biological component of a functional circadian clock is output pathways, or the ability to connect the timekeeping oscillator with the control of gene expression to manifest overt biological rhythms (Figure 1-1). In cyanobacteria, a prokaryote, nearly the entire genome was under the control of the circadian clock through regulation of the bacterial chromosome compaction (Golden et al., 1997). In the fungus Neurospora, a simple eukaryotic circadian model organism, the expression of around 20% of the genome was under the control of the clock at the level of transcript abundance (Vitalini et al., 2006). In the mouse, a smaller percentage, around 10%, of the transcriptome was under control of the circadian clock, and the identity of rhythmic transcripts varied

between tissue types (Storch et al., 2002). The oscillator is thought to control rhythmic output using many different mechanisms. The simplest mechanism is for oscillator components themselves to act as direct regulators of gene expression (Koike et al., 2012; Smith et al., 2010). While the clock does directly control the expression of many gene products, many of the direct targets of the oscillator are transcriptional regulators or signaling components, implying a network of clock influenced genes that extends beyond the direct clock targets (Smith et al., 2010) (Figure 1-1). Dissecting the intricacies of the output pathways has been underappreciated as a vital function of the circadian clock. It is somewhat straightforward to identify either direct targets of the oscillator or gene products that are rhythmic. However, the ability to methodically dissect a complete regulatory network seems to be shrouded by the redundancy and complexity of cellular signaling networks. Furthermore, designating a gene as a clock output is complicated by the fact that some output genes, for example *vivid* in Neurospora (Chen et al., 2010; Smith et al., 2010), or NAMPT and SIRT1 in the mouse (Ramsey et al., 2009), feedback onto the oscillator, and therefore, they can act as an input to the circadian clock.

MAPK SIGNALING PATHWAYS

As one of the well conserved signaling pathways in eukaryotes, the mitogen activated protein kinase (MAPK) pathway, has been shown to function as both an output and input pathway in circadian clock (Bennett et al., 2013; de Paula et al., 2008; Dziema et al.,

2003; Wang and Sehgal, 2002). The MAPK pathway relays extracellular signals from outside the cell, as well as intracellulary within the cytoplasm, to mediate an appropriate cellular response to a given mitogen (Roux and Blenis, 2004) (Figure 1-3). The MAPK signaling pathway functions as a kinase cascade that is composed of a canonical three tier hierarchy of serine/threonine kinases. At the very top tier, the MAPK kinase kinase (MAPKKK) is activated by cellular signaling components in response to a stimulus. Once active, the MAPKKK phosphorylates an associated MAPK kinase (MAPKK). Phosphorylation induces conformational changes that mediate binding with its target MAPK. Finally, the activated phospho-MAPKK couples with its cognate MAPK, the terminal component of the cascade (Roux and Blenis, 2004). Once the MAPK is phosphorylated, it can associate with a host of cellular factors to control gene expression and various other cellular processes (Roux and Blenis, 2004). Multiple MAPK pathways belonging to distinct sub-families are present in nearly all eukaryotic organisms and exist in parallel (Johnson and Lapadat, 2002). Three families of MAPK pathways have emerged: extracellular signal regulated kinase (ERK's), c-Jun NH_2-terminal kinase (JNK), and p38 (Johnson and Lapadat, 2002) (Figure 1-3). While it simplifies matters to think of each MAPK pathway as being insulated and linear, evidence reflects a much different reality. Many of the components of signaling pathways are shared between families. This relationship seems to increase the more upstream in a cascade that a particular component lies (Johnson and Lapadat, 2002). How a vast network of interconnected components relays and integrates information with specificity is an unknown question that will likely rely heavily on mathematics,

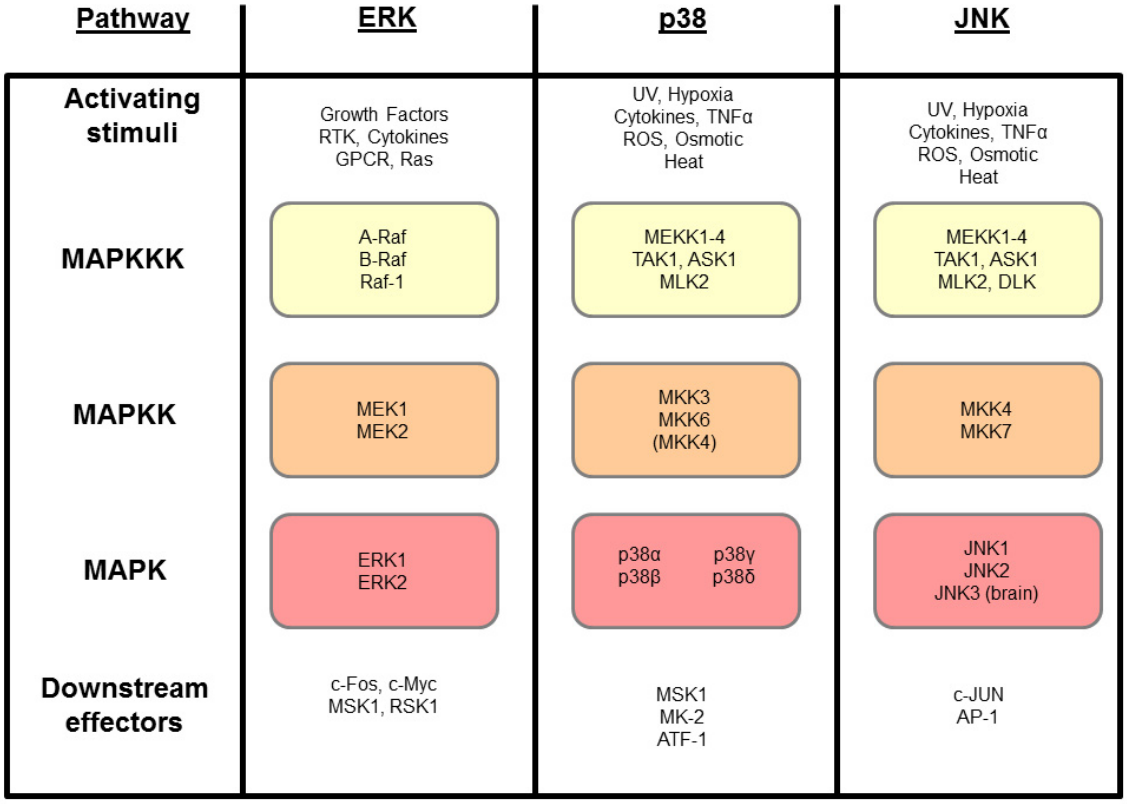


Figure 1-3. Organization of MAPK pathways in mammals. Each MAPK family is organized to show the canonical signaling cascade in mammals. Activating stimuli induce the activity of the MAPKKK, which propagates a signal through the MAPKK, and finally, to the MAPK. Downstream effectors further propagate the signal to relevant molecules.

computational, and systems biology approaches to demystify. However, MAPK pathways use molecular scaffolds as one means to achieve signaling specificity (Morrison and Davis, 2003; Whitmarsh and Davis, 1998). Scaffolds can physically co-localize proteins in a cascade, while excluding components of parallel MAPK pathways, in order to mediate a specific signal. Feedback inhibition, in which the activity of a MAPK leads to the inhibition of its own components or the components of a neighboring pathway, is a mechanism that similarly contributes to signaling fidelity (Kolch, 2005; O'Rourke and Herskowitz, 1998). Often times, this inhibition is achieved through regulation of phosphatases that dephosphorylate and, therefore, inactivate MAPK's (Owens and Keyse, 2007). Additionally, MAPK pathways can have many different functions depending on the cell type (Zarubin and Han, 2005).

ERK MAPK PATHWAY

The ERK MAPK's are a well characterized MAPK pathway and are typified by the classic example of ERK1 and ERK2 in mammals (Roux and Blenis, 2004) (Figure 1-3). In general, these kinases signal in response to growth factors, or to a lesser degree, stress signals. Receptor tyrosine kinases (RTK's) and GPCR's at the cell surface, activate Ras complex, the most potent activator of ERK1/2 MAPK cascade. Ras activates the Raf proteins that act as MAPKKK's in the ERK pathway (Roux and Blenis, 2004). These MAPKKK's signal to the MAPKK MEK1 and MEK2 and finally activate ERK1/2 (Figure 1-3). ERK1 and ERK2 show 83% amino acid identity, and are also frequently

co-activated. Activation of the ERK MAPK's is facilitated by dual phosphorylation of a Thr-Glu-Tyr motif. Downstream targets of ERK1/2 include cancer-related substrates, such as c-Fos and c-Myc, as well as a host of interacting regulatory kinases such as MSK-1 and RSK-1 (Figure 1-3). Thus, it is not surprising that ERK's have been shown to play an important role in the regulation of cell growth and proliferation as its major upstream activator, Ras, is frequently aberrantly activated in cancer (Davies et al., 2002).

ERK MAPK IN THE CIRCADIAN CLOCK

Role of ERK in light-induced clock-resetting in the vertebrate SCN

The function of ERK's in circadian signaling was most thoroughly investigated in neural tissue, and ERK's were found to play a role in the process of photic clock-resetting in the rodent SCN (Obrietan et al., 1998). In order for the SCN clock to fulfill its role as a master pacemaker, the clock is entrained by light via direct innervation from the eyes. Photosensitive retinal ganglia cells (pRGC) in the retina detect light through the blue light photoreceptor melanopsin (Schmidt et al., 2011). These neurons projected directly to the SCN through the retinohypothalamic tract (RHT) where they release glutamate and pituitary adenylate cyclase-activating peptide (PACAP) that are ligands for NMDA and Pac1 receptors, respectively, at post-synaptic SCN neurons (Dibner et al., 2010) (Figure 1-4). These neurotransmitters facilitate an increase in intracellular calcium that, along with Ras/ERK activation, culminate in the activation of CRE-binding protein

(CREB) (Lonze and Ginty, 2002). Activation of CREB is mediated through phosphorylation at Serine-133. The Ser-133 phosphorylation site is a target of a myriad of kinases, including protein kinase C (PKC), protein kinase A (PKA)/ adenosine 3',5'-monophosphate (cAMP), neural specific calmodulin-dependent kinase IV (CaMKIV), as well as ERK MAPK's (Dziema and Obrietan, 2002; Schurov et al., 2002) (Figure 1-4). Activation of CREB in response to light, which was partly dependent on the activity of phosphorylated-ERK (p-ERK), stimulated transcriptional activation through CRE cis-elements on the promoters of target genes (Lonze and Ginty, 2002; Obrietan et al., 1998) (Figure 1-4). The immediate early genes (IEG) are targets of CREB that are upregulated following photic stimulation in the SCN (Ginty et al., 1993). Many of these genes, notably c-Fos and mPer1, required a functional ERK pathway for their light-induced expression (Dziema et al., 2003). mPer1 is a clock gene in the negative arm of the circadian oscillator, and its induction is thought to be a primary event in the resetting of the clock (Tischkau et al., 2003). Consistent with a role for ERK MAPK in light signaling to the clock, infusion of ERK inhibitor into the mouse SCN prevented phase shifts in locomotor rhythms when given during the subjective night (Butcher et al., 2002). Furthermore, the activation of ERK was phase-gated (Obrietan et al., 1998), occurring only at the subjective night, and the magnitude of a phase shift was proportional to levels of active ERK (Butcher et al., 2003).

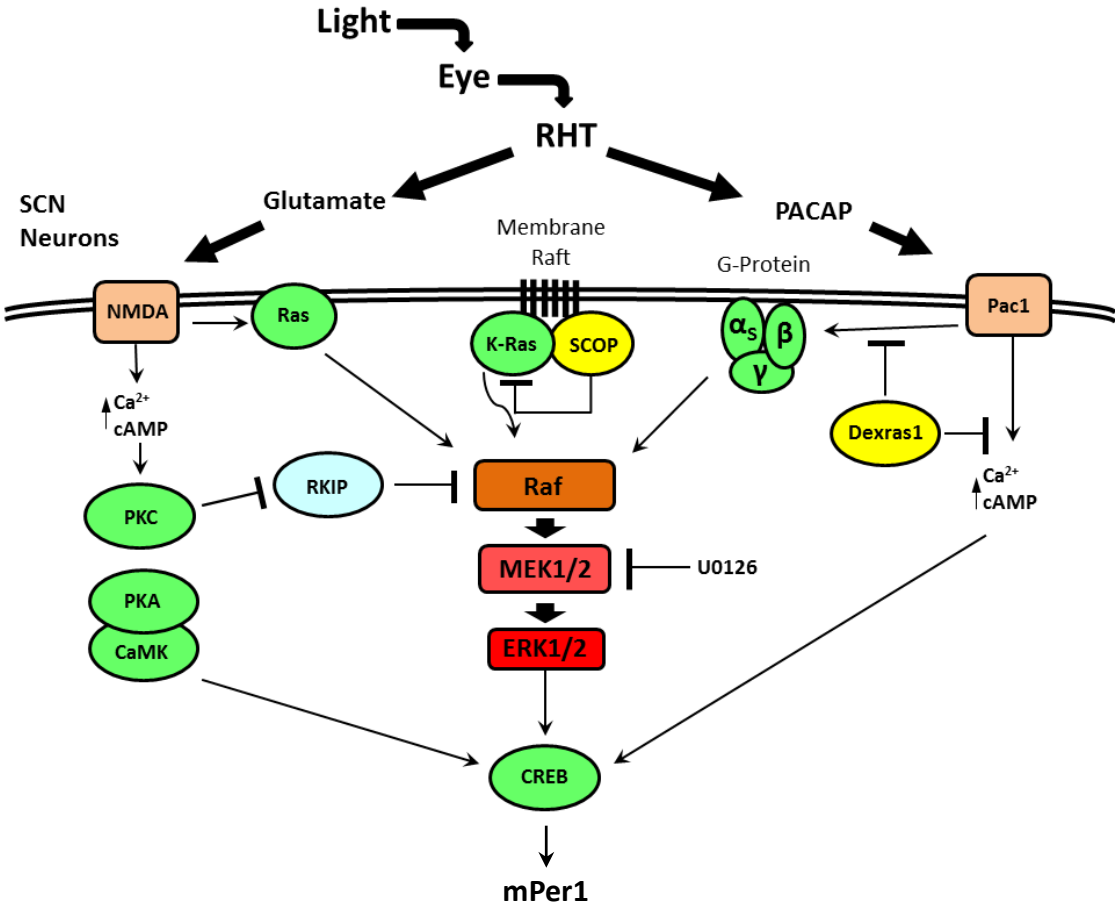


Figure 1-4. Diagram of the light responsive ERK signaling network in the SCN. In light-mediated clock resetting, the neurotransmitters glutamate and PACAP are released onto SCN neurons via the eye and RHT. The NMDA and Pac1 receptors lead to the activation of ERK through Ras protein and heterotrimeric G-proteins, and proteins like RKIP, SCOP, and Dexras1, as well as the pharmacological inhibitor U0126, modulate the activation of ERK. The activation of ERK in response to light, along with parallel signaling pathways, leads to the activation of CREB, which is responsible for the induction of the immediate early genes, including mPer1. Components that are rhythmically expressed are shown in yellow.

Endogenous rhythm of p-ERK in the SCN

Light-induced activation of ERK is important for the resetting of the mammalian circadian oscillator in response to light; however, the activation of ERK also has an endogenous rhythm in the SCN (Obrietan et al., 1998; Pizzio et al., 2003). While the phosphorylation state of ERK, and thereby its activity, displayed a circadian rhythm, ERK protein levels remained constitutive, indicating that clock regulation of ERK activation was post-translational (Obrietan et al., 1998). Interestingly, the p-ERK activation rhythm varied between different anatomical regions of the SCN (Nakaya et al., 2003; Obrietan et al., 1998). The dorsomedial sector of the SCN, known as the shell, cycled with an apex of ERK activation during the subjective day, the phase of the oscillator cycle that would coincide with daytime in an LD cycle (Nakaya et al., 2003; Obrietan et al., 1998). In contrast, the neurons located in the ventrolateral region of the SCN, known as the "core", cycled with a peak in p-ERK during the subjective night (Nakaya et al., 2003; Obrietan et al., 1998). While it is apparent that rhythmic activity of the ERK pathway is under control of the circadian clock, the function of the ERK activity rhythm is not yet known. The SCN core receives direct afferents from the RHT (Lee et al., 2003; Nakaya et al., 2003). However, in enucleated mice, no expression of p-ERK was seen in the SCN core, while p-ERK continued to cycle in the SCN shell (Lee et al., 2003), indicating that rhythms in the SCN shell are not dependent on the SCN core. Consistent with the idea that the core receives neural connections directly from the retina, night-time photic stimulation only resulted in p-ERK induction in the SCN core

(Nakaya et al., 2003). Thus, the endogenous rhythms in ERK activation are segregated into distinct anatomical regions of the SCN, but the functional relevance of segregation is not understood.

In order to generate rhythmic ERK MAPK activation, a gene called SCN Circadian Oscillatory Protein (SCOP) was proposed to provide a mechanism for the endogenous rhythmicity of ERK activation in the SCN (Shimizu et al., 1999; Shimizu et al., 2003). SCOP was rhythmically expressed in the SCN with a peak in abundance during the late night (Shimizu et al., 1999). Within the cell, SCOP localized to a membrane raft and bound to the K-Ras isoform through a leucine rich repeat (LRR) domain (Shimizu et al., 2003) (Figure 1-4). Through this protein-protein interaction, SCOP bound to the nucleotide-free form of K-Ras, thereby inhibiting the association of K-Ras with GTP, leaving Ras inactive (Shimizu et al., 2003). Consistent with this hypothesis, *in vitro* expression of SCOP inhibited activation of ERK (Shimizu et al., 2003). In this manner, SCOP is proposed to act as a mechanism to generate endogenous rhythms in ERK activation, through rhythmic inhibition of the upstream Ras, during the subjective day. However, it is unknown whether SCOP is selectively expressed in distinct anatomical regions of the SCN, such as the shell and core, or if SCOP contributes to the phase difference of p-ERK rhythms in these two regions.

The modulation of light-induced ERK activation

Due to the rapidity of the photic response on p-ERK, and the fact that the ERK protein levels do not fluctuate, it is clear that the mechanism that generates light induction of ERK activity is post-translational (Obrietan et al., 1998). As mentioned previously, the ERK MAPK lies downstream of the monomeric G-protein Ras and heterotrimeric G-proteins (Roux and Blenis, 2004). An upstream regulatory component called Dexras1 is responsible for the phase gating property of ERK activation in response to photic stimulus (Cheng et al., 2004). Dexras1, a rhythmically expressed Ras-like G-protein in the SCN with a peak in expression during the subjective night, blocked the activity of PACAP, a neuropeptide responsible for light-induced phase advances in the late night (Cheng et al., 2004; Cheng et al., 2006) (Figure 1-4). Through the inhibition of $\beta\gamma$ and α_S subunits of heterotrimeric G-proteins that signal through PACAP receptors, Dexras1 was able to block both p-ERK and cAMP induction that resets the clock in the night time. Also, Dexras1 positively enhanced p-ERK in response to light-induced NMDA gluatamertergic signaling, which elicits a phase delay (Cheng et al., 2006). Although the role that Dexras1 has in the clock mechanism is not entirely clear, Dexras1-null mice show defects in entrainment, have phase response curves with exaggerated phase shifts, and phase shift in response to daytime light (Cheng et al., 2006).

In order modulate ERK pathway activation following a light stimulus, the protein Raf Kinase Inhibitory Protein (RKIP) regulates the activation of the MAPKK in the ERK

pathway (Figure 1-4). In the absence of a light stimulus, RKIP is bound to the Raf MAPKKK in the mouse SCN, thereby preventing activation of the downstream MAPKK (Yeung et al., 1999). Following a light stimulus, the PKC pathway is activated and mediates the phosphorylation of the RKIP protein. PKC-mediated phosphorylation of RKIP protein led to its dissociation from the Raf complex and relieved the suppression of the upstream MEK MAPKK activation (Corbit et al., 2003). This mechanism couples ERK activation to PKC signaling, which is co-activated in the SCN in response to photic stimulation (Antoun et al., 2012). RKIP modulated ERK activation so that activation following light input was appropriately transient (Antoun et al., 2012). $RKIP^{-/-}$ mice exhibited prolonged p-ERK in response to a light stimulus, as well as prolonged Per1 and c-Fos transcription, and exaggerated phase shifts during subjective night (Antoun et al., 2012). Alternatively, RKIP mutation had no effect on the endogenous rhythm of p-ERK in the SCN indicating that RKIP modulates the response of p-ERK to light, but not the clock in the SCN (Antoun et al., 2012). Through these regulatory mechanisms, the parallel PKC signaling pathway is able to modulate the persistence of ERK activation in response to light by inhibiting the upstream Raf kinase.

ERK MAPK in other neural tissues

The SCN is not the only neural tissue in which the ERK MAPK acts as a vital signaling pathway for the circadian clock. The pineal gland, a neuroendocrine component of the clock that contributes to overall rhythmicity of the organism, produces the hormone

melatonin that is secreted at night into the circulatory system and relays temporal information to peripheral oscillators (Dibner et al., 2010; Ho et al., 2003). In the rat pineal gland, the ERK pathway is activated by adrenergic receptors that bound norepinephrine from the sympathetic nervous system (Ho et al., 2003). Through this neural stimulation, the ERK pathway is rhythmic in an LD cycle, with a peak in activation during the night. This activity of ERK contributes to the production of a rate-limiting precursor in melatonin production (Ho et al., 2003). In contrast to the SCN, direct photic stimulation of the pineal gland resulted in suppression of p-ERK as well as a simultaneous decrease in melatonin precursor, suggesting that p-ERK connects neural stimulation to the production of melatonin (Ho et al., 2003). A couple of reports have described a similar role for ERK in the chick pineal gland (Hayashi et al., 2001; Sanada et al., 2000); however, a conflicting report from Yadav and colleagues (Yadav et al., 2003) indicated that ERK did not play a role in rhythmic melatonin synthesis in the chick pineal gland. Technical differences between the two groups' methodology likely explain the conflicting results. Furthermore, in chick retina, ERK is rhythmically phosphorylated with its apex during subjective night (Ko et al., 2001). This rhythm was important in modulating photoperception in the retina (Ko et al., 2001). In the chick retina, the ERK pathway, along with CaMKII, modulates the membrane potential of photoreceptive cone cells through cGMP-gated cation channels, so as to regulate the photosensitivity of neurons based on the time of day (Ko et al., 2001). ERK was also shown to be involved in the clock regulation of L-type voltage gated calcium channels that facilitate the rhythmic production of melatonin in photoreceptor cells (Ko et al.,

2007). Interestingly, the circadian regulation of p-ERK was dominant to photic stimulation in chick retina, and its activation state was solely dependent on circadian phase (Ko et al., 2009). Together, these data demonstrate that the ERK pathway couples environmental stimuli in neural tissue to coordinate circadian rhythms in the whole organism.

ERK-mediated regulation of the circadian oscillator

ERK MAPK can directly interact with components of the circadian oscillator, and ERK-mediated phosphorylation of these proteins likely plays an important role in the maintenance and resetting of biological rhythms (Akashi and Nishida, 2000; Sanada et al., 2002; Sanada et al., 2004; Weber et al., 2006) (Figure 1-5). Through several studies with *in vitro* systems, ERK has been shown to interact with clock components (Sanada et al., 2002; Sanada et al., 2004; Weber et al., 2006). In yeast, the chicken ERK protein interacted with Bmal1, a positive component of the molecular oscillator, regardless of its activation state (Sanada et al., 2002). Mass spectrometry identified several Bmal1 phosphorylation sites that are targets of ERK, and these phosphorylation sites facilitate the repression of CLOCK/BMAL transactivation (Sanada et al., 2002). Likewise, the Drosophila CLOCK gene, which heterodimerizes with CYCLE to form the positive component of the Drosophila circadian feedback loop (Hardin, 2005), can be phosphorylated by ERK2 in cultured cells (Weber et al., 2006). The negative clock components of the mammalian clock, mCry1 and mCry2, also directly interacted with

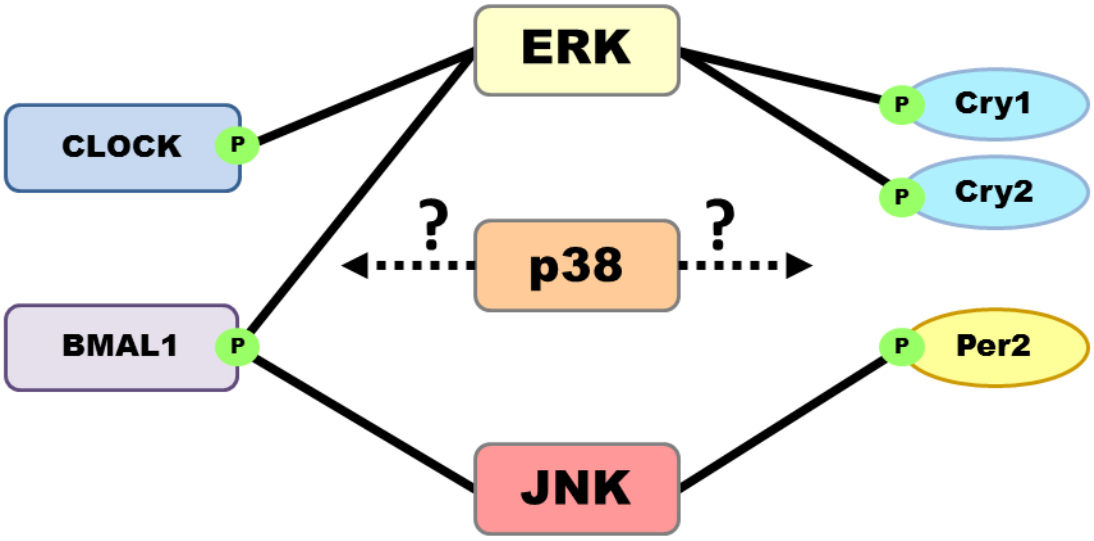


Figure 1-5. Interaction of MAPK's with clock proteins. MAPK's from different families can directly influence clock proteins in the molecular oscillator. ERK MAPK physically interacts with and phosphorylates CLOCK and BMAL1 in the positive branch of the oscillator, and CRY1 and CRY2 in the negative branch. No physical interactions of p38 with clock proteins have been observed, and no p38-specific phosphorylation sites have been identified on clock proteins. However, inhibition of p38 led to a longer period of clock gene oscillation, implying that p38 may have an unknown effect on the molecular oscillator. Finally, JNK MAPK interacts with and phosphorylates BMAL1 and PER2.

ERK in COS7 cells, and phosphorylation sites on both mCry1 and mCry2 were determined by mass spectrometry to be specific targets of ERK (Sanada et al., 2004) (Figure 1-5).

Given that ERK can directly phosphorylate the gene products of the molecular oscillator, one might expect manipulation of ERK activity to cause defects in endogenous circadian rhythms. Interestingly, chronic defects in ERK signaling did not lead to defects in biological rhythms (Antoun et al., 2012; Butcher et al., 2003). In the whole mouse, administration of the ERK inhibitor U0126 had no effect on the free running locomotor rhythm of mice in constant conditions (Butcher et al., 2002). However, the authors conceded this result could be due to a short half-life of the drug that was infused into the brain only once (Butcher et al., 2002). A transgenic mouse with a constitutively active allele of RKIP constantly suppressed ERK signaling in the SCN, but no defect in its free running locomotor rhythm was observed (Antoun et al., 2012). In a more direct approach, cultured mouse SCN were bathed with the ERK inhibitor U0126 leading to a rapid decrease in amplitude of Per2 protein rhythms, as well as a decrease in overall Per2 protein levels (Akashi et al., 2008). The persistence of Per2 rhythmicity while ERK is inhibited can be explained by the fact that Per2 expressing neurons in the SCN were not necessarily ERK-active cells. This suggests that Per2 can be rhythmically expressed in neurons where ERK is not active, and therefore, ERK may support clock gene expression through intercellular communication rather than direct regulation (Akashi et al., 2008). It is possible that the disruption of ERK signaling is negligible

towards oscillator function since both positive and negative components of the oscillator can be ERK targets, thereby offsetting its effect. On the other hand, as the mechanism of ERK-mediated clock-resetting relies on transcriptional activation of the downstream immediate early genes, including Per1 (Dziema et al., 2003) the major role of ERK-mediated phosphorylation of clock components appears to be in light-induced clock-resetting.

ERK signaling through downstream components

ERK MAPK has an array of downstream effector molecules that in turn regulate the expression of target genes. These targets can be transcription factors, kinases, or even translational regulators (Roux and Blenis, 2004) (Figure 1-6). The transcription factor Elk-1 is downstream of ERK and, its activity, regulated by its ERK-dependent phosphorylation, leads to transcriptional activation of target genes (Davis et al., 2000). Phosphorylated Elk-1 (p-Elk-1) is induced via glutamate treatment, and also bound the serum response element (SRE) in the promoter of target genes that include c-Fos and Per1 (Davis et al., 2000; Vanhoutte et al., 1999) (Figure 1-6). While p-ERK cycled with an endogenous rhythm, p-Elk-1 was not rhythmic in the SCN (Coogan and Piggins, 2003). However, Elk-1 was phosphorylated in response to a light pulse given at night, and Elk-1 required a functional ERK pathway for this phosphorylation (Coogan and Piggins, 2003). These data suggested that Elk-1 is a downstream regulator of ERK in response to photic stimulation in the SCN (Coogan and Piggins, 2003). Bioinformatic

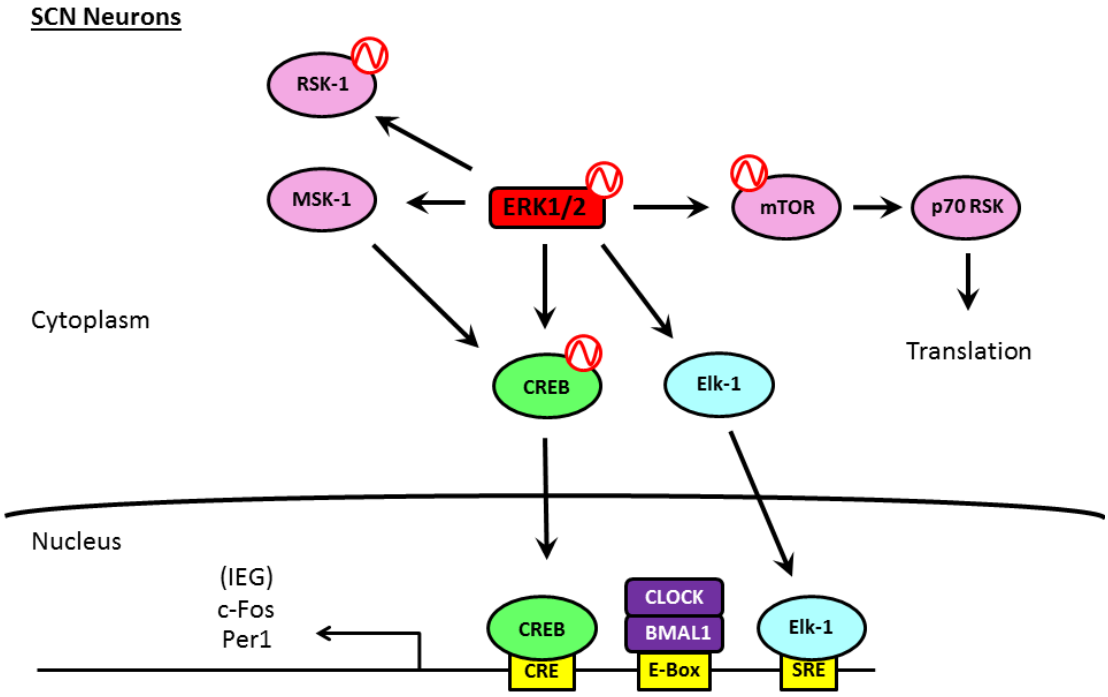


Figure 1-6. Downstream components of ERK in SCN neurons. Activated ERK can signal through various downstream components in the SCN. The intermediate kinases RSK-1 and MSK-1 are activated by ERK after light stimulus. Also, ERK signals through the mTOR/p70 RSK pathway to putatively regulate translation of target genes. Finally, ERK activates the CREB and Elk-1 transcription factors to induce the expression of the immediate early genes in response to light stimulus. Rhythmicity in the activity of a protein is indicated by a red sine wave.

analysis in mouse liver has shown that the SRE is enriched in the promoters of clock-controlled genes (ccg's) (Bozek et al., 2010). Thus, given the established role of Elk-1 as a transcription factor downstream of ERK, it is likely that Elk-1 has an important role in regulating ERK target genes in the SCN in response to light, and through rhythmic activation of ERK.

In addition to Elk-1, ERK interacts with a network of downstream effector kinases to regulate diverse cellular processes (Anjum and Blenis, 2008; Roux and Blenis, 2004) (Figure 1-6). For example, the p90 ribosomal S6 kinase 1 (RSK-1) is an ERK-regulated kinase that requires a functional ERK pathway for light-induced phosphorylation in the SCN (Butcher et al., 2004). Also, RSK-1 has a circadian rhythm in phosphorylation that correlates with the p-ERK endogenous rhythm (Butcher et al., 2004). Notably, phosphorylated RSK-1 co-localized with ERK-active neurons only in the SCN core, not in the shell, in response to circadian ERK activation (Butcher et al., 2004). This observation indicated that ERK uses specific effector kinases in different anatomical regions of the SCN (Butcher et al., 2004). Another intermediate effector kinase, mitogen and stress activated protein kinase 1 (MSK-1), acted as an intermediate between ERK and CREB transcriptional activation in cortical neurons after stimulation with neurotrophins (Arthur et al., 2004). In the mouse SCN, active phosphorylated MSK-1 co-localized with p-ERK staining cells after a light pulse, or after administration of PACAP, during the subjective night (Arthur et al., 2004). Interestingly, MSK-1 did not show a circadian rhythm in activation, and may function as a downstream target of ERK

only in response to photic stimulation (Arthur et al., 2004). In support of this hypothesis, a transgenic MSK-1 knockout mouse given a light pulse demonstrated diminished phase shifts in response to photic stimulation, as well as lower levels of necessary gene products for clock-resetting: phosphorylated CREB (p-CREB), c-Fos, and Per1 (Cao et al., 2013).

The ERK MAPK also couples with downstream processes that are unrelated to transcriptional activation. Through micro-ribonucleic acid (miRNA) expression, the ERK/CREB signaling module can regulate gene expression at the post-transcriptional level. miR-132 is a light-induced miRNA in the mouse SCN that requires a functional ERK pathway and CREB signaling (Cheng et al., 2007). Blockage of miR-132 activity led to reduced phase-shifting after a light pulse (Cheng et al., 2007), and miR-132 was shown to enhance Per1 protein expression after a light pulse (Cheng et al., 2007). Also, the mammalian target of rapamycin pathway (mTOR) is a master regulator of translation and plays a significant role in the SCN clock (Cao et al., 2008; Cao et al., 2010; Cao et al., 2011) (Figure 1-6). The activity of mTOR is rhythmic in the SCN (Cao et al., 2011), and inhibition of mTOR activity led to defective light-induced phase-shifting of locomotor rhythms (Cao et al., 2010). The mTOR-regulated kinase p70 RSK was activated in the SCN after a light pulse, and strongly co-localized with the subset of ERK active neurons in the SCN (Cao et al., 2008). Similarly, inhibition of either ERK or mTOR suppressed light-induced p70 RSK activation (Cao et al., 2010). These data indicated that an ERK/mTOR/p70 RSK signaling pathway can regulate translation in

response to photic stimulation in the SCN (Figure 1-6). Because p70 RSK activity following a light pulse co-localized with p-CREB, investigators hypothesized that the ERK was coordinating transcriptional activation and the translational machinery to upregulate the expression of light-responsive genes (Cao et al., 2008). However, a role for the kinases and transcription factors that function downstream of ERK in the control of ccg's is not well defined.

ERK in peripheral tissues

Limited data exists for ERK having a role in the circadian clock of peripheral tissues in mammals, and insights from model organisms will be useful in forming hypothesis in more complex systems. The role for the ERK MAPK in the circadian clock is most clearly defined in the SCN, and because the function of the SCN is to transduce environmental stimuli into the generation of autonomous biological rhythms, ERK has been strongly implicated as part of the input pathway to the circadian oscillator. This suggests that the ERK pathway might also function as an input pathway to cellular circadian oscillators in peripheral tissues. Individual cells within a tissue need to synchronize their clocks to both external time and to maintain synchrony with other cells in a tissue through signals from the master pacemaker SCN (Mohawk et al., 2012). For example, the circadian clock in cultured fibroblasts could be reset by treatment with dexamethasone, forskolin, or concentrated serum, all of which induced common signaling pathways as photic stimulus in the SCN, and leading to the activation of the

same immediate early genes (Balsalobre et al., 2000). For example, the Z3 cell line from zebrafish is derived from peripheral tissue, and retains the ability to detect light (Cermakian et al., 2002). Photic stimulation induced the expression of the clock gene zPer2 in the Z3 cell line, and pharmacological inhibition of ERK blocked the light induction of zPer2 (Cermakian et al., 2002).

ERK as an output pathway

Although output pathways frequently exert some feedback onto the oscillator (Roenneberg and Merrow, 1998), in general, output pathways relay temporal information from the timekeeping oscillator to terminal target genes. This definition assumes that disruption of an output pathway will disrupt rhythmicity of ccg's without affecting the function of the oscillator. If ERK activity rhythms are maintained in peripheral tissues, it would seem logical to assume that in this respect, ERK is acting as an output pathway, as opposed to in the SCN where the function of that tissue is to reset the clock and act as a master pacemaker. Examples of ERK MAPK rhythms in peripheral tissue are few, however, rhythmic ERK activity has been reported in mouse liver (Tsuchiya et al., 2013).

In mammalian neural tissue, ERK MAPK appears to act as a circadian output pathway in mouse hippocampus to modulate memory formation (Eckel-Mahan et al., 2008; Luo et al., 2013; Phan et al., 2011; Shimizu et al., 2007). CREB-mediated transcription, along

with cAMP and ERK MAPK signaling, plays an important role in memory formation within the hippocampus (Athos et al., 2002; Atkins et al., 1998; Blum et al., 1999; Bourtchuladze et al., 1994; Pittenger et al., 2002; Sindreu et al., 2007; Wu et al., 1995). In the hippocampus, p-ERK cycled with an endogenous rhythm with its apex during subjective night when mice were typically sleeping (Eckel-Mahan et al., 2008), and these rhythms were dependent on an intact SCN (Phan et al., 2011). When rhythmicity of p-ERK was disrupted by housing mice in constant light (LL), the mice showed a deficit in the ability to consolidate, or store, long term memories (Eckel-Mahan et al., 2008; Phan et al., 2011). Over-expression of SCOP, which suppressed ERK activation through K-Ras inhibition (Shimizu et al., 2003), impaired the ability of mice to consolidate long term memories (Shimizu et al., 2007). Interestingly, further temporal resolution of p-ERK expression during subjective night showed that increases in p-ERK were only observed in rapid eye movement (REM) sleep, but not during non-REM sleep (Luo et al., 2013). REM sleep is known to be an important stage in the sleep cycle for memory formation (Louie and Wilson, 2001; Poe et al., 2000), and therefore, strongly implicates the ERK MAPK as a causative mechanism for memory formation.

More abundant evidence exists in model organisms to understand how ERK functions in output pathways. For example, the *neurofibromatosis-1* (*nf*-1) gene in Drosophila is a Ras-GTPase whose mutation abolishes activity rhythms in DD (Williams et al., 2001). Clock gene messenger RNA (mRNA) rhythmicity was not affected by *nf-1* mutation, and ectopic expression of functional *nf-1,* specifically in the lateral neurons, the locus of the

master oscillator in Drosophila, did not rescue the behavioral phenotype (Williams et al., 2001). Only expression of functional *nf-1* in the whole organism was able to rescue rhythmic behavioral rhythms (Williams et al., 2001). These data indicate that the mutation of *nf-1* disrupts the circadian output pathway, and does not affect the activity of the endogenous circadian oscillator. The levels of the *rolled* MAPK, the ERK homologue in Drosophila, were significantly higher in the *nf-1* mutants, and a double mutant of the *nf-1* mutation combined with loss-of-function mutations in the MAPK pathway rescued rhythmic behavioral rhythms (Williams et al., 2001). These results confirmed that the defect in behavioral rhythms is mediated through the ERK MAPK pathway. Additionally, staining of fly brains from flies kept in LD cycles revealed a rhythm in ERK MAPK activation (Williams et al., 2001). The staining of p-ERK co-localized with pigment-dispersing factor (PDF), a rhythmic neuropeptide responsible for activity rhythms, in the fly brain (Williams et al., 2001). Due to the co-localization of p-ERK with PDF, it was hypothesized that Ras/ERK acts downstream of PDF to regulate rhythmic behavior (Williams et al., 2001). In accordance with the definition of an output pathway, it was observed that, unlike mammalian cells, disruption of the Ras/ERK pathway has no effect on the function of the circadian oscillator in DD (Williams et al., 2001). In this model, the GTPase *nf-1* functions to regulate rhythmic Ras/MAPK activity, that in turn, leads to circadian changes in behavioral rhythms (Williams et al., 2001).

The ERK-related MAPK's in the fungus Neurospora, called MAK-1 and MAK-2, are rhythmically activated in constant conditions by the circadian clock (Bennett et al., 2013). As is characteristic of an output pathway, mutation of these MAPK's did not disrupt the endogenous rhythmicity of the oscillator (Bennett et al., 2013). Transcriptional profiling was performed in rhythmic cultures of either wild type or Δ*mak-1* strains during the subjective morning to identify potential target genes of MAK-1 (Bennett et al., 2013). Around 28% of the 517 putative MAK-1 targets were predicted to be ccg's, giving insight into the proportion of target genes that are rhythmically expressed from the circadian activity of a MAPK (Bennett et al., 2013).

Summary

Predominantly, the ERK pathway acts as a circadian input pathway that relays a light activated signal from the eyes to molecular oscillator in SCN pacemaker neurons (Butcher et al., 2003; Obrietan et al., 1998) (Figure 1-4). Through the induction of p-CREB and IEG's, ERK mediates resetting of the molecular oscillator (Butcher et al., 2002; Dziema et al., 2003). Additionally, ERK is able to phosphorylate many clock proteins in the molecular oscillator; however, the biological significance of these phosphorylation events in not known (Akashi and Nishida, 2000; Sanada et al., 2002; Sanada et al., 2004; Weber et al., 2006) (Figure 1-5). The activity of ERK cycles with a circadian rhythm in the mouse SCN through a mechanism proposed to involve the SCOP protein (Pizzio et al., 2003; Shimizu et al., 2003). Activated ERK signals through the

transcription factors CREB and Elk-1, the intermediate kinases MSK-1 and RSK-1, and the mTOR pathway to regulate gene expression of its targets (Arthur et al., 2004; Butcher et al., 2004; Cao et al., 2008; Coogan and Piggins, 2003; Dziema et al., 2003) (Figure 1-6). In some neural tissues, such as the retina and pineal gland, ERK functions as an output pathway that can regulate the production of melatonin or feedback onto input pathways to phase-gate environmental sensing (Ho et al., 2003; Ko et al., 2001; Ko et al., 2009). Finally, in model organisms, ERK is utilized as an output pathway by the clock to regulate rhythmic gene expression and behavioral rhythms (Bennett et al., 2013; Williams et al., 2001).

Future studies can address many unknown areas of ERK signaling in the circadian clock. Since ERK functions as a vital input pathway for clock-resetting in SCN pacemaker neurons (Obrietan et al., 1998), a similar role in peripheral tissues needs to be examined. Rhythmic ERK activity is known to phase-gate input pathways in the retina (Ko et al., 2001; Ko et al., 2007); therefore, it is possible that ERK may regulate the ability of peripheral oscillators to reset as well. While ERK phosphorylates clock proteins *in vitro* (Akashi and Nishida, 2000; Sanada et al., 2002; Sanada et al., 2004; Weber et al., 2006), the significance of this phosphorylation *in vivo* is unknown. It will be important to determine in what manner ERK phosphorylates clock proteins. For example, does ERK phosphorylate clock proteins in response to environmental stimuli, implying a function in clock-resetting, or perhaps rhythmically in accordance with its endogenous rhythm, implying an interlocked feedback loop with the molecular oscillator? Additionally, it

will be important to determine the effect that ERK-specific phosphorylation has on the biochemistry of clock proteins. Interestingly, there are no examples of rhythmic ERK activity in mammals outside of the brain, but based on the tendency of the clock to use this pathway as an output in model organisms, a similar role for ERK is likely to exist in mammals. Nonetheless, ERK activity rhythms may have interesting effects in specific areas of the brain. ERK activity rhythms have been observed in the hippocampus and correlated with the ability to form memories (Eckel-Mahan et al., 2008). Additionally, an increase in ERK activity has been shown to confer resistance to glutamate-induced neurotoxicity (Karmarkar et al., 2011). This suggests that rhythms in ERK activity may confer an increased resistance to naturally occurring incidences of neurotoxicity, such as stroke or brain damage, at certain times of the day.

P38 MAPK PATHWAY

Similar to the ERK MAPK, the p38 MAPK is activated by dual phosphorylation of conserved Tyr-Gly-Thr residues (Zarubin and Han, 2005). There are four known isoforms of p38 (α, β, γ, and δ) that show tissue specific expression, however, p38α is the most ubiquitously expressed isoform (Zarubin and Han, 2005) (Figure 1-3). The MAPK is tightly regulated by the MAPKK genes MKK3 and MKK6, although some signaling through MKK4, the JNK related MAPKK, does occur (Zarubin and Han, 2005). A large network of MAPKKK's feed into the p38 MAPK, including MEKK1-4, TAK1, ASK1, and MLK2 (Roux and Blenis, 2004) (Figure 1-3). The most classic

activating signal of the p38 pathway is through the inflammatory cytokine tumor necrosis factor α (TNFα) and lippopolysacharride (LPS) (Roux and Blenis, 2004). p38 MAPK mediates the inflammatory response, especially through cytokines secreted from immune cells (Zarubin and Han, 2005). Additionally, p38 is activated by many stress signals, such as reactive oxygen species (ROS), osmotic stress, and heat shock (Zarubin and Han, 2005). Deoxyribonucleic acid (DNA) damage also activates p38 MAPK, and in turn regulates many genes related to cell cycle arrest and apoptosis (Zarubin and Han, 2005). It is of particular note that the role of p38,and the expression of its different isoforms, are variable in different cell types. Thus, the p38 MAPK pathway provides a mechanism to address cell-type specific needs (Zarubin and Han, 2005).

The p38 MAPK pathway as a potential circadian input pathway in neural tissue

In contrast to the ERK MAPK, p38's role in clock-resetting is less clear and appears for the most part dispensable (Arthur et al., 2004). Most investigations have explored whether p38 has a similar biochemical role as ERK, or if it acts in an accessory role. In one study, p38 MAPK was shown to be rhythmically phosphorylated in the hamster SCN with a peak in the late afternoon or early subjective night in both LD and DD conditions, similar to ERK (Pizzio et al., 2003) (Figure 1-7). Additionally, p38 MAPK was activated in response to light, but only during the subjective night, again, matching the phase gating of ERK in the hamster SCN (Pizzio et al., 2003). In cultured rat pineal gland, the activation of p38 MAPK was very similar to ERK in that both were rhythmic

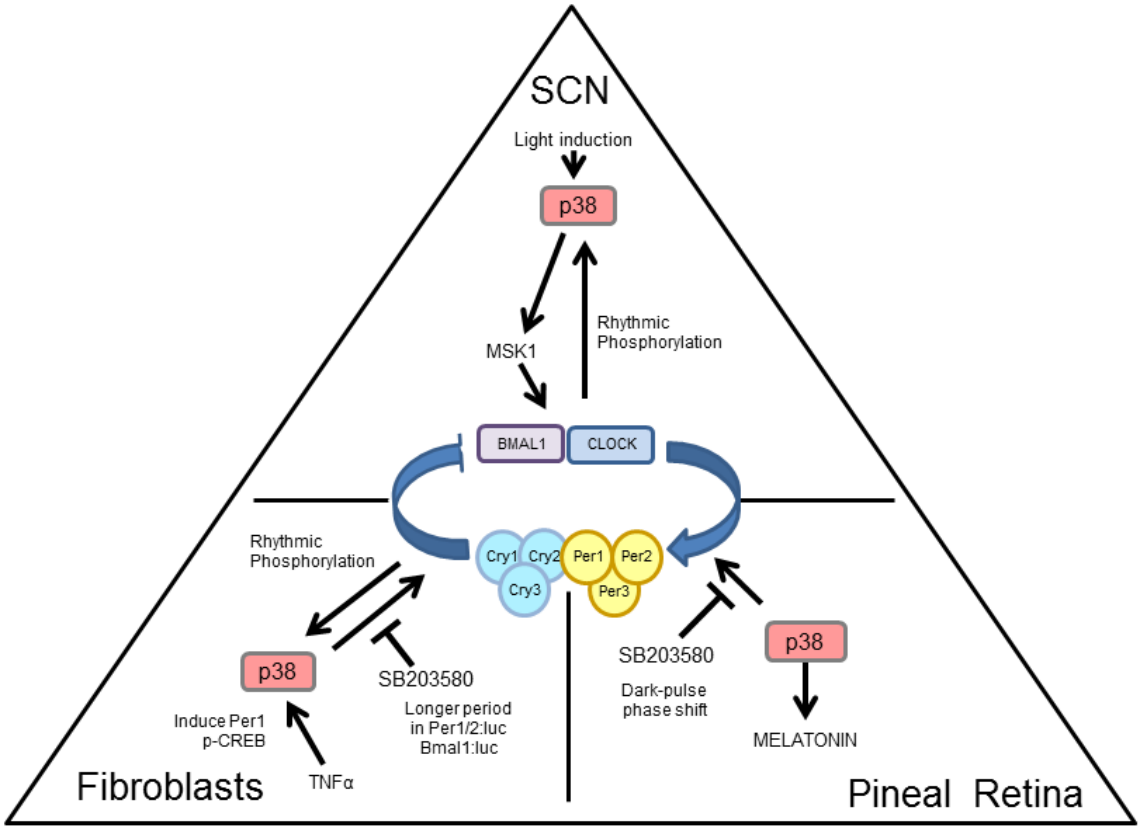


Figure 1-7. The role of p38 MAPK in the circadian clock. The diagram presents the most representative tissues in mammals with regards to the function of p38: either SCN, fibroblasts, or the pineal/retina. The oscillator in the center of the triangle represents the cellular oscillator in the respective tissues. The arrows that are drawn toward the oscillator represent a circadian input pathway. The lines that are drawn away from either the oscillator or p38 MAPK represent output pathways. The labels indicate the functions of pathways or perturbations on the activity of p38.

in a LD cycle with peaks during the subjective night (Chik et al., 2004). Again similar to ERK, administration of norepinephrine, the hormone produced by the sympathetic nervous system that acts on adrenergic receptors in the pineal gland, led to an increase in phosphorylated p38 (p-p38), while a light pulse at night led to a decrease in p-p38 (Chik et al., 2004). However, there was a noticeable difference in the kinetics of enzymatic activation/deactivation between ERK and p38 in response to these stimuli (Chik et al., 2004). In response to norepinephrine, ERK was maximally phosphorylated at 30 min, while p38 reached its peak in activation at 60 min (Chik et al., 2004). Light at night led to a more gradual dephosphorylation of p38 MAPK compared to ERK, and, therefore, p38 was unlikely to be involved in the output pathway that regulated melatonin biosynthesis with ERK, since light repression of melatonin occurred on a much quicker time scale (Chik et al., 2004). These data demonstrated that the p38 MAPK is often co-activated with ERK, but p38 remains active over a prolonged time frame in the pineal gland.

In cultured Xenopus retina, the p38 MAPK appeared to play a role in photic resetting of the clock-controlled rhythm in melatonin production (Hasegawa and Cahill, 2004). Administration of the p38 inhibitor SB 203580 had no acute effect on the production of melatonin, but brief treatments of cultured retinas with SB 203580 led to phase shifts in the melatonin rhythm (Hasegawa and Cahill, 2004) (Figure 1-7). These phase shifts resembled dark pulses, meaning that treatment during the day led to a phase delay, whereas treatment during the night led to a phase advance (Hasegawa and Cahill, 2004).

In the chick pineal gland, another tissue that rhythmically produces melatonin, brief SB 203580 treatment also generated a dark pulse-like phase response (Hayashi et al., 2003; Yadav et al., 2003) (Figure 1-7). Together, these data suggested that p38 does not signal in the output pathway that regulates melatonin rhythms, but instead, p38 is part of an input pathway that affects the ability of the endogenous oscillator to cycle. Treatment of Xenopus retina with p38 inhibitor in combination with light pulses yielded phase shifts that were strangely different from treatment with either inhibitor or light alone. However, the authors demonstrated that SB 203580 had non-specific interactions with other kinases, such as JNK MAPK and CKIε, that could account for the confusing phase shifts after inhibitor and light pulse treatment (Hasegawa and Cahill, 2004). As a validation of this hypothesis, JNK inhibition with the drug SP600125 also resulted in dark pulse-like phase shifts of the melatonin rhythm, and CKIε-specific inhibition led to phase shifts, although they were not dark pulse-like shifts (Hasegawa and Cahill, 2004). CKIε has a well documented role as a modulator of the circadian oscillator (Kloss et al., 1998; Lowrey et al., 2000; Price et al., 1998), and the off target effects of the SB 203580 inhibitor on a combination of kinases likely contributed to the phase shifting effects of the drug. Indeed, the lack of specificity of p38 inhibitors, specifically SB 203580 and SB 202190, have been thoroughly examined (Fabian et al., 2005) and show a significant non-specific interaction with casein kinases. Given these non-specific interactions, data generated from the usage of p38 inhibitors to test properties of the circadian clock should be carefully interpreted. Thus, while the data indicate a conserved role for the p38 MAPK pathway in circadian input in neural tissues, additional studies are needed.

The p38 MAPK pathway is a circadian input pathway in peripheral tissues

The mammalian p38 MAPK pathway is thought to impinge upon the circadian oscillator after activation by the inflammatory cytokine TNFα (Petrzilka et al., 2009; Zarubin and Han, 2005) (Figure 1-7). TNFα in humans has been implicated in daytime fatigue (Spriggs et al., 1988), and conversely, inflammatory disease-associated fatigue seems to be suppressed by treatment with TNFα antagonists (Pollard et al., 2006; Vgontzas et al., 2004). To investigate a putative role for TNFα in circadian disruption, mouse fibroblasts were used to measure the circadian response to TNFα. TNFα induced Per1 expression via p38 activation; although, TNFα treatment was not sufficient to induce endogenous circadian rhythms (Petrzilka et al., 2009). The upregulation of Per1 through CREB-mediated transcription is a critical event in clock-resetting, and treatment of fibroblasts with p38 inhibitor SB 203580 effectively blocked TNFα-mediated induction of both p-CREB and Per1 (Petrzilka et al., 2009). These data suggested that p38 is involved in clock-resetting after potent activation by TNFα.

The p38 MAPK has also been implicated in circadian input that couples humoral signals from the SCN to individual oscillators in peripheral tissues (Ko et al., 2011). Wild type adult mice maintain a rhythm in p-p38 within cardiac tissue when housed in an LD cycle (Ko et al., 2011). The cardiac-specific expression of a dominant null allele of *Clock* abolished the circadian oscillator in cardiomyocytes, but peripheral oscillators outside of the heart remained intact (Bray et al., 2008). In mice with cardiac-specific disruption of

the circadian oscillator, p38 MAPK maintained diurnal activation rhythms in the heart, indicating that p38 may be relaying a rhythmic extracellular signal from the SCN, even while the heart lacks a functional oscillator (Ko et al., 2011). However, in this study, there was no experimental control to rule out light- or activity-driven rhythms of p-p38 in the heart. Although the p38 MAPK can induce clock gene expression in response to cytokines, it is unclear if p38 receives signals from the SCN to reset peripheral oscillators.

The p38 MAPK affects the endogenous rhythmicity of the circadian oscillator

While several examples support that p38 MAPK relays signals to facilitate clock-resetting, the activity of p38 also appears to affect the circadian oscillators' ability to maintain endogenous rhythms (Figure 1-7). In the chick pineal gland, chronic treatment with the SB 203580 inhibitor lengthened the endogenous period of melatonin production (Hayashi et al., 2003). A similar period lengthening effect was described in mouse cell lines. In U2OS cells, treatment with p38 inhibitor SB 202190 lengthened the *Bmal1:luc* rhythm by 1 hr. Similarly, in the C6 mouse rhythmic glioblastoma cell line, treatment with both p38 inhibitors SB 203580 and SB 202190 increased the period length of an *mPer2:luc* transcriptional reporter, although the intensity of bioluminescence was severely diminished (Yagita et al., 2009). Similar to the ERK MAPK, p38 may phosphorylate clock oscillator proteins, thereby affecting the stability of these proteins and altering endogenous circadian rhythmicity. However, another possibility is that the

increase in period was due to non-specific interaction with targets like CKIε that are known to phosphorylate clock proteins, and whose inhibition led to longer circadian periods (Fabian et al., 2005; Isojima et al., 2009; Kloss et al., 1998; Lowrey et al., 2000; Meng et al., 2010; Price et al., 1998). The p38 inhibitor VX-745 may be better suited to study the circadian effect of p38 inhibition due to its high degree of potency and specificity, and its lack of off target action on casein kinases (Fabian et al., 2005).

The p38 MAPK acts as a circadian output pathway

There is no data demonstrating a role for p38 MAPK as a circadian output pathway in mammals, but model organisms have once again proved their usefulness in studying p38. The OS-2 MAPK in Neurospora, the p38 homologue, is rhythmically phosphorylated in constant conditions with a peak during the early subjective morning, and this rhythm required a functional circadian oscillator (Vitalini et al., 2007). Importantly, mutation of *os-2* had no effect on the endogenous rhythms of the circadian oscillator, demonstrating that the OS-2 MAPK functions as an output pathway between the oscillator and target genes (Vitalini et al., 2007). The upstream OS-4 MAPKKK is a direct target of the positive component of the Neurospora circadian oscillator, the White Collar Complex (WCC), and is rhythmically expressed through direct transcriptional activation (Lamb et al., 2011). Interestingly, the circadian rhythm of phosphorylated OS-2 (p-OS-2) is dependent on the rhythmicity of the upstream OS-4 MAPKKK, suggesting that transcriptional activation of MAPKKK is a mechanism to generate endogenous rhythms

in MAPK activation (Lamb et al., 2011). Consistent with the prediction that circadian activation of OS-2 in early subjective morning prepares the organism to anticipate daily environmental stresses, tissue challenged with an osmotic stress during the subjective morning mounted a more robust adaptive response compared to tissue treated during the subjective night (Lamb et al., 2011). Presumably, this time-of-day difference is mediated via ccg's that are regulated by the OS-2 MAPK (Noguchi et al., 2007; Watanabe et al., 2007). Analysis of the downstream transcription factor ASL-1, the homologue to mammalian ATF-1, revealed that the rhythmicity of several ccg's induced by OS-2 activation required ASL-1 for endogenous circadian rhythmicity (Lamb et al., 2012). However, not all genes under control of the OS-2/ASL-1 pathway were ccg's, which suggested that other mediators are responsible for selectively conferring rhythmicity to ASL-1 target genes (Lamb et al., 2012).

Summary

Similar to the ERK MAPK pathway, p38 appears to have a role as an input pathway to the circadian clock. p38 MAPK was activated by light in the hamster SCN during the subjective night and was rhythmically activated in DD (Pizzio et al., 2003). In the pineal gland and retina, inhibition of p38 MAPK led to phase shifts (Hasegawa and Cahill, 2004; Hayashi et al., 2003; Yadav et al., 2003), and treatment of fibroblasts with the cytokine TNFα led to p38 dependent induction of clock gene expression (Petrzilka et al., 2009). Treatment of mammalian cell lines or chick pineal glands with p38 inhibitors

produced longer oscillations in clock gene expression and melatonin production, respectively, suggesting that p38 MAPK is able to phosphorylate clock proteins (Hayashi et al., 2003; Yagita et al., 2009). Finally, in the model organism Neurospora, the p38-like MAPK is a circadian output pathway that allows the organism to anticipate daily stress by providing a more robust response at a specific times of the day (Lamb et al., 2011; Lamb et al., 2012; Vitalini et al., 2007).

In future studies, the function of p38 as a circadian input pathway should be more clearly defined. While the p38 MAPK was rhythmically activated and light-induced in the hamster SCN (Pizzio et al., 2003), no functional role of p38 in clock-resetting or entrainment has been established in the rodent SCN. Interestingly, p38 MAPK mediated clock gene expression in fibroblasts in response to TNFα (Petrzilka et al., 2009), but was unable to generate rhythmic clock gene expression. This indicates that, while p38 is involved in the circadian response to TNFα, it is not sufficient to completely reset the clock. This result does raise the interesting possibility that p38 is an important modulator of circadian rhythms in immune cells. Furthermore, p38 inhibition led to a longer period of circadian rhythms implying that p38 can phosphorylate clock proteins (Hayashi et al., 2003; Yagita et al., 2009). It will be important to determine which clock genes and which sites are phosphorylated by p38. Also, further studies will be necessary to determine the function of phosphorylation at those sites. Given the fact that the p38 MAPK pathway serves as a circadian output in the model organism Neurospora (Lamb et al., 2011; Vitalini et al., 2007), further studies should determine if p38 is rhythmically

activated in peripheral oscillators in mammals. Because the p38 pathway is a stress response pathway in both Neurospora and animals (Zarubin and Han, 2005), it is likely that the clock utilizes this pathway to anticipate daily stress in animals.

JNK MAPK PATHWAY

The c-Jun NH_2-terminal kinase (JNK) is activated by phosphorylation of the canonical MAPK activation motif Thr-Pro-Tyr (Barr and Bogoyevitch, 2001; Roux and Blenis, 2004). Of the three JNK MAPK genes, JNK1 and JNK2 are expressed in most cells, whereas JNK3 is expressed mainly in the brain (Roux and Blenis, 2004) (Figures 1-1 & 1-3). These three JNK MAPK's do not have redundant signaling roles, however, and sometimes oppose each other's activity (Bogoyevitch, 2006). The JNK MAPK's are activated by two upstream MAPKK's, MKK4 and MKK7 (Barr and Bogoyevitch, 2001). The top tier of the JNK cascade shares many MAPKKK's with p38 ((Roux and Blenis, 2004) (Figure 1-3). Given the commonality in the upstream MAPKKK network with p38, it is not surprising that JNK is activated by many of the same stimuli as p38, with a particular sensitivity to stress signals and cytokines (Barr and Bogoyevitch, 2001). However, in order to maintain specificity of downstream signaling events in spite of the regulators in common with p38, the scaffold JNK interacting protein 1 (JIP-1) physically associates the components of the JNK pathway (Barr and Bogoyevitch, 2001; Whitmarsh and Davis, 1998). The most recognized downstream target of the JNK MAPK's are Jun proteins involved in the activating protein 1 (AP-1) transcriptional activating complex

(Hess et al., 2004). In contrast to the other MAPK subfamilies, JNK MAPK is not known to signal to any downstream intermediate kinases (Roux and Blenis, 2004).

The JNK MAPK's modulate the circadian oscillator

With regards to a function in the circadian clock, there are currently no reports demonstrating a role for JNK MAPK's in output pathways. The existing studies showed that JNK kinase acted as an input to the circadian clock by phosphorylating clock oscillator proteins (Chansard et al., 2007; Yoshitane et al., 2012) (Figure 1-5). Initial studies showed that the JNK inhibitor SP600125 lengthened the period of melatonin production in chick pineal gland and bullfrog retina (Bennett et al., 2001; Hasegawa and Cahill, 2004; Hayashi et al., 2003) (Figure 1-8). In the hamster and mouse SCN, a light pulse at night led to the phosphorylation of JNK (Pizzio et al., 2003; Yoshitane et al., 2012). Additionally, in rat-1 fibroblasts, a media change that reset the clock also led to the phosphorylation of JNK MAPK (Chansard et al., 2007). Also, endogenous rhythms in JNK activation were observed in both hamster SCN and rat-1 fibroblasts (Chansard et al., 2007; Pizzio et al., 2003). In order to gauge the effect of JNK on oscillator function, the rhythms of clock genes were analyzed in several different cell lines after treatment with the SP600125 inhibitor. In rat-1, NIH-3T3, and C6 mouse glioblastoma cell lines, treatment with JNK inhibitor led to a longer period, ranging from 31-45 hours, of clock gene oscillations (Chansard et al., 2007; Yagita et al., 2009; Yoshitane et al., 2012). Interestingly, treatment of rat-1 fibroblasts with valproic acid, which induces

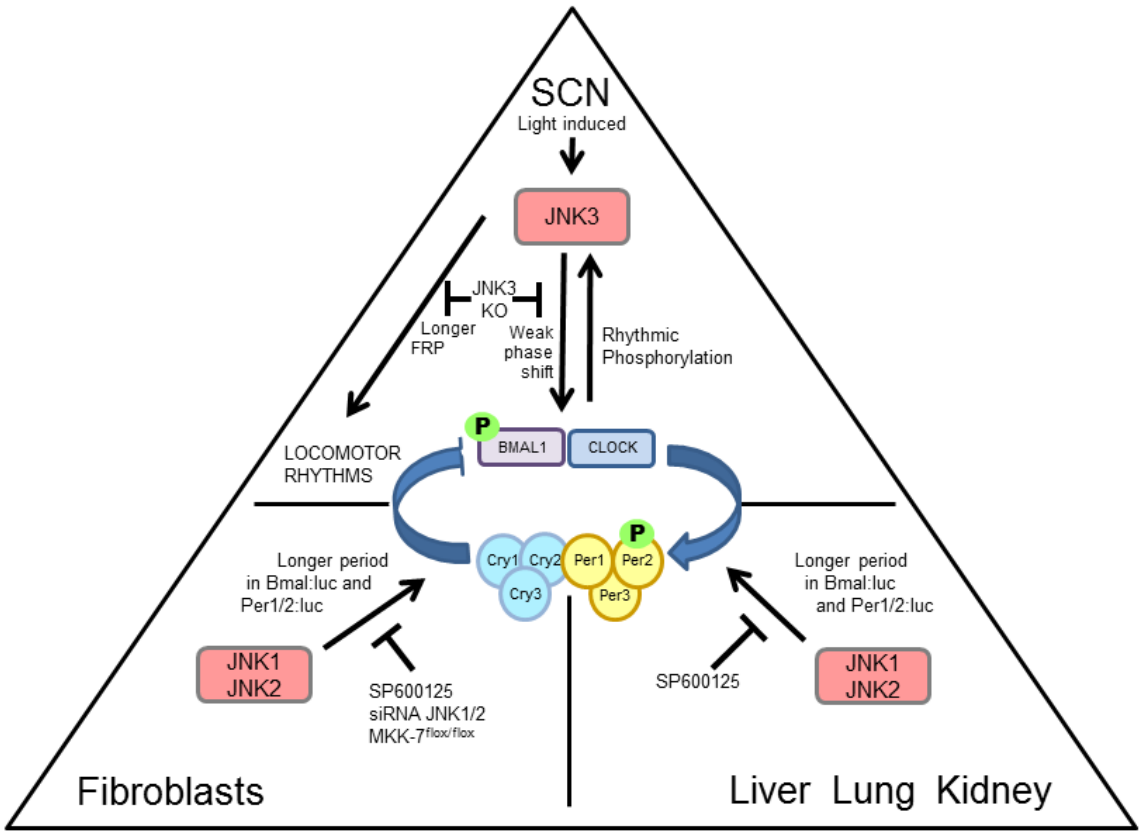


Figure 1-8. The role of JNK MAPK in the circadian clock. The diagram presents the most representative tissues in mammals with regards to the function of JNK: the SCN, fibroblasts, or liver, lung, and kidney. The oscillator in the center of the triangle represents the cellular oscillator in the respective tissues. The arrows that are drawn toward the oscillator represent a circadian input pathway. The lines that are drawn away from either the oscillator or JNK MAPK represent output pathways. The labels indicate the functions of pathways or perturbations on the activity of JNK.

phosphorylated JNK (p-JNK), showed a 1 hour shorter period of *Per1:luc* transcriptional rhythms (Chansard et al., 2007). These data taken together indicated that the activity of JNK contributed to a faster oscillation of the molecular clock. Furthermore, mouse organs in explant culture treated with JNK inhibitor caused clock gene oscillations to exhibit longer periods (Chansard et al., 2007) (Figure 1-8). Mouse SCN, pineal, and lung explant cultures had longer period oscillations from *Per1:luc* and *Per2:luc* transcriptional reporters that were dose dependent on SP600125 and reversible after drug washout (Chansard et al., 2007). Unfortunately, like the p38 inhibitors, SP600125 affects a broad range of other kinases, including CKI (Bain et al., 2003; Fabian et al., 2005). Fortunately, several genetic disruptions have clarified the effects of JNK activity on clock gene oscillations. Knockdown of JNK1/2 expression by siRNA in NIH-3T3 cells led to a longer period of *Bmal1:luc* (1-2 hours longer), whereas a conditional knockout of MKK7, the upstream MAPKK, using the $Mkk7^{flox/flox}$ allele, had a similar period defect in *Per2:luc* in synchronized mouse embryonic fibroblasts (MEF's) (Uchida et al., 2012; Yoshitane et al., 2012) (Figure 1-8). Finally, a JNK3 knockout mouse, which lacked the brain-specific isoform of JNK kinase and presumably abolished signaling through this MAPK in the SCN, was assayed for locomotor rhythms (Yoshitane et al., 2012). In the JNK3 knockout mouse, circadian locomotor rhythms in DD had a 1-2 h longer period when compared to wildtype (Yoshitane et al., 2012). These mice also responded to a light pulse given during the subjective night with a much diminished phase shift, indicating a defect in circadian entrainment (Yoshitane et al., 2012). Interestingly, the JNK3 knockout did not have any effect on the light-induced

transcription of Per1 in the SCN, suggesting that the JNK MAPK does not participate in CREB-mediated Per1 activation. As a whole, these data suggested that JNK functions within an input pathway to the circadian clock that is responsive to clock-resetting stimuli, and can modulate the period of the oscillator.

JNK kinase modulated the properties of the circadian oscillator by phosphorylating clock proteins in both the positive and negative branches of the oscillator (Uchida et al., 2012; Yoshitane et al., 2012) (Figure 1-5). Bmal1 protein, which forms the positive component of the mouse oscillator upon heterodimerization with CLOCK, was hyperphosphorylated following over-expression of a hyperactive MKK7-JNK fusion protein, but not with a "kinase dead" fusion protein in NIH-3T3 cells (Yoshitane et al., 2012). Furthermore, knock down of JNK MAPK in NIH-3T3 cells using siRNA decreased the phosphorylation state of Bmal1, in addition to lengthening the period of a *Bmal1:luc* reporter (Yoshitane et al., 2012). Similarly, co-expression of the hyperactive MKK7-JNK fusion with Bmal1 in 293T cells increased the phosphorylation state of Bmal1 (Uchida et al., 2012), and co-immunoprecipitation of Bmal1 with JNK MAPK in 293T cells indicated interaction between the two proteins *in vitro* (Uchida et al., 2012). In the negative branch of the circadian oscillator, JNK MAPK phosphorylated PER2. The dynamics of PER2 phosphorylation play an important role in modulation of period and other properties of the oscillator (Vanselow et al., 2006; Xu et al., 2007). Similar to BMAL1, PER2 was phosphorylated by the hyperactive MKK7-JNK fusion in 293T cells, and endogenous PER2 was found in association with JNK MAPK using co-

immunoprecipitation in synchronized MEFs (Uchida et al., 2012) (Figure 1-5). Importantly, the phosphorylation of PER2 by JNK had a significant effect on protein levels and stability. In 293T cells, expression of the MKK7-JNK fusion, but not the kinase dead allele, increased the levels of PER2 protein by enhancing its stability (Uchida et al., 2012). Consistent with these data, expression of the hyperactive JNK fusion in MEF's decreased the ubiquination of PER2, indicating that JNK activity led to an increase in stability and protein levels of PER2 by decreasing its degradation via the proteasome (Uchida et al., 2012). JNK-mediated stabilization of PER2 is consistent with the period lengthening effect of JNK kinase in circadian clock gene oscillations. This particular property of JNK activity was tested by contrasting it with the period shortening effect of CKIε-mediated phosphorylation (Eide et al., 2005; Uchida et al., 2012). Over-expression of CKIε in 293T cells destabilized PER2 protein, but co-expression of the MKK7-JNK fusion protein reduced the CKIε-mediated effect on PER2 stability (Uchida et al., 2012). Interestingly, the activity of JNK and CKIε did not compete for the same phosphorylation site, but instead, catalyzed the phosphorylation of unique phosphorylation sites (Uchida et al., 2012). These studies suggested that the mechanism of JNK-mediated modulation of the circadian oscillator relied on phosphorylation of clock proteins, which in turn affected clock protein stability and subcellular localization.

Summary

The current understanding of JNK MAPK indicates a role in circadian input through phosphorylation of clock proteins (Uchida et al., 2012; Yoshitane et al., 2012) (Figure 1-5). In the rodent SCN, activation of JNK MAPK was induced by a light pulse, and JNK displayed a circadian rhythm in its activation (Chansard et al., 2007; Pizzio et al., 2003) (Figure 1-8). Consistent with a role in input pathways, a disruption of the brain-specific JNK isoform led to diminished phase shifts in locomotor behavior after light pulses (Yoshitane et al., 2012). Interestingly, this defect in phase shifting was independent of Per1 induction, the classical mechanism of clock-resetting in the SCN (Yoshitane et al., 2012). Furthermore, disruption of JNK activity led to a lengthening of circadian rhythms in clock gene oscillations in mammalian cell culture and organs in explant culture, as well as circadian rhythms in locomotor behavior (Chansard et al., 2007; Yagita et al., 2009; Yoshitane et al., 2012). The effect that JNK has on circadian rhythms is a likely the result of its interaction with clock proteins in the molecular oscillator. JNK MAPK has been found to associate with and to phosphorylate the clock proteins BMAL1 and PER2 (Uchida et al., 2012; Yoshitane et al., 2012). Importantly, JNK-mediated phosphorylation of PER2 was shown to increase the stability of this protein (Uchida et al., 2012).

Future studies should clarify the mechanism of JNK on the modulation of circadian rhythms. JNK-mediated phosphorylation of PER2 increased protein stability (Uchida et

al., 2012), but no specific function of JNK-mediated phosphorylation of BMAL1 has been demonstrated. Furthermore, a circadian rhythm of JNK activity was observed in both pacemaker and peripheral tissues (Chansard et al., 2007; Pizzio et al., 2003; Uchida et al., 2012). Further investigations should determine if the rhythm in JNK activation is a circadian output that feeds back onto the molecular oscillator. While the JNK MAPK has an important role in modulating the molecular oscillator, the AP-1 transcriptional activating complex appears to be dispensable in this process, as JNK directly phosphorylates clock proteins. Future studies should investigate whether downstream components of the JNK pathway have an effect on the clock. Because the AP-1 is the classical target of the JNK pathway (Hess et al., 2004), it should be investigated whether a rhythmic signal is relayed to AP-1 through the JNK MAPK. This would imply a potential role for the JNK pathway in circadian output through the regulation of gene expression.

SUMMARY OF MAPK FUNCTION IN CIRCADIAN CLOCKS

Through their ability to relay biochemical signals within the cell and in response to extracellular stimuli, MAPK pathways are important components of the circadian clock. Phosphorylation of clock proteins by all three MAPK subfamilies is apparent in diverse eukaryotic organisms; however, the biological significance of clock protein phosphorylation by MAPK's is poorly understood. While the role of MAPK's as circadian input pathways is firmly established, a role for these signal transduction

pathways in circadian output is only beginning to emerge. The activity of MAPK's is frequently regulated by the clock, but this rhythmic activation is rarely connected to known downstream signaling components. The rhythmic interaction of MAPK's with downstream components will expand the understanding of MAPK's ability to rhythmically regulate gene expression and translational control. Finally, as the role of the circadian clocks is more thoroughly understood in peripheral tissues, MAPK's will likely be contributors to circadian rhythms due to their ability to assume unique functions in disparate tissues.

NEUROSPORA AS A MODEL TO STUDY THE CONNECTION BETWEEN THE CLOCK AND MAPK PATHWAYS

As demonstrated in the literature, model organisms can be a powerful tool to dissect molecular phenomena that is shared by the conserved nature of the circadian clock in eukaryotes (Dunlap and Loros, 2004). The filamentous fungus *Neurospora crassa* has a well-defined circadian oscillator, and its unicellular nature, combined with it numerous genetic tools, makes it an amenable model to understand generic properties of the circadian clock (Dunlap and Loros, 2004). Additionally, its robust oscillations in asexual spore production are an easily measurable output of the circadian clock (Sargent et al., 1966). This phenotype was used to screen for mutations that altered the clock of *N. crassa* (Gardner and Feldman, 1980), and led to the cloning of several of the first clock genes (Dunlap et al., 1995). The gene *frequency* (*frq*) was the first clock gene in

Neurospora to be identified (Gardner and Feldman, 1980), and several mutant alleles were isolated with defective period lengths (Feldman and Hoyle, 1973). The genes WC-1 and WC-2, which heterodimerize to form the WCC, were identified a short time later as clock the clock genes responsible for light entrainment (Crosthwaite et al., 1997; Merrow et al., 2001).

Consistent with oscillators in other eukaryotic organisms, the clock genes in *N. crassa* form a transcriptional-translational negative feedback loop (Dunlap, 1999). The WCC, the positive component in the oscillator, is a transcription factor and blue-light photoreceptor, and after heterodimerization, it activates transcription of the *frq* gene through binding of *frq* promoter (Cheng et al., 2001b; Crosthwaite et al., 1997; Froehlich et al., 2003a). *frq* mRNA accumulates, and after a brief delay, is subsequently translated into protein (Aronson et al., 1994; Garceau et al., 1997). As FRQ protein accumulates, it forms a complex with *FRQ-interacting RNA helicase* (*frh*) (Cheng et al., 2001a; Cheng et al., 2001b) and recruits several kinases, including CKI and CKII (He et al., 2006; Yang et al., 2001; Yang et al., 2002; Yang et al., 2003; Yang et al., 2004). The FRQ:FRH complex, with associated kinases, is imported into the nucleus and physically interacts with the WCC (Denault et al., 2001; Luo et al., 1998; Merrow et al., 1997). Upon interaction with the WCC, the FRQ:FRH-associated kinases inactivate the WCC by phosphorylation, thereby inhibiting the expression of *frq* (He et al., 2005; He et al., 2006). Over time, the FRQ protein accumulates phosphorylation mediated by its associated kinases (Baker et al., 2009), which leads to the instability of FRQ protein.

Progressive phosphorylation leads to degradation via SCF^{FWD-1} ubiquitin ligation. As FRQ is degraded, its repression of the WCC is diminished, and the cycle can restart the next day (He et al., 2003; He et al., 2006).

Input into the oscillator is primarily relayed by the light-responsive WCC (Cheng et al., 2003; Crosthwaite et al., 1995; Froehlich et al., 2002; He and Liu, 2005b). As a blue-light photoreceptor, the WCC activates a large network of transcriptional targets in response to a light stimulus (Smith et al., 2010). However, the WCC also activates a partially overlapping network of genes through its light-independent function as a component of the circadian oscillator (Smith et al., 2010). Interestingly, many of these WCC targets are themselves transcription factors, revealing a broad network of transcriptional targets under the regulation of the clock (Smith et al., 2010). In other studies, many ccg's have been identified that have functions relating to stress response, metabolism, and structural support (Bell-Pedersen et al., 1992; Bell-Pedersen et al., 1996). One of these genes involved in stress response, *ccg-1*, is regulated by the p38- and ERK- like MAPK pathways (Bell-Pedersen et al., 1996; Bennett et al., 2013; Vitalini et al., 2007). Given that all three MAPK pathways in *N. crassa* are clock regulated, and that around 20% of the genome is influenced by the clock, *N. crassa* is an excellent organism to study the function of MAPK pathways in the regulation of circadian output.

In this document, studies are presented to understand the basis of MAPK signaling in circadian output pathways. In Neurospora, the p38-like MAPK, OS-2, acts as an output

of from the circadian oscillator to control the expression of ccg's. The molecular mechanism generating endogenous circadian rhythms in OS-2 activation is partially defined. In this mechanism, the circadian clock regulated the expression of upstream regulatory components of OS-2 to generate phosphorylation rhythms of the OS-2 MAPK. Furthermore, clock regulation of the OS-2 MAPK contributes towards a more robust physiological adaptation after exposure to stress. As an extension of the work in Neurospora, the p38 MAPK is found to be rhythmically phosphorylated in two mammalian cell lines that model functionally relevant tissues in the mouse. The p38 MAPK phosphorylation rhythm is shown to be dependent on a functional circadian oscillator. Overall, this work contributes to the field of circadian biology by demonstrating a molecular mechanism utilized by the circadian clock to generate endogenous rhythms in MAPK activation. Furthermore, the biological significance of circadian rhythms in stress response pathways is defined. Finally, for the first time circadian rhythms in p38 MAPK activation are demonstrated in a mammalian peripheral tissue.

CHAPTER II

DIRECT TRANSCRIPTIONAL CONTROL OF A P38 MAPK PATHWAY BY THE CIRCADIAN CLOCK IN *Neurospora crassa*[*]

INTRODUCTION

Eukaryotic cells adapt their physiology to respond to changing extracellular conditions for enhanced fitness and survival. Two complex mechanisms exist in cells to deal with environmental conditions: adaptation and anticipation. When the environment changes in unpredictable ways, conserved stress-induced MAPK signaling pathways are activated to promote adaptation (Waskiewicz and Cooper, 1995). The acute response mobilized by the MAPK pathways can occur at any time of day to improve survival (Vitalini et al., 2007). When the environment changes on a predictable basis, such as the day/night cycle, an internal timing mechanism called the circadian clock provides the machinery to anticipate the change and program daily rhythms in gene expression to prepare for daily stresses. In this way, the clock provides an adaptive advantage to organisms (Dodd et al., 2005; Woelfle et al., 2004). Because these two mechanisms can be directed to cope with

[*] Reprinted with permission from "Direct Transcriptional Control of a p38 MAPK Pathway by the Circadian Clock in *Neurospora crassa*" by Lamb, T.M., Goldsmith, C.S., Bennett, L., Finch, K.E., and Bell-Pedersen, D., 2011. *PLoS One* ,6(11): e27149; reproduced under a Creative Commons Attribution License.

similar stressors, for example light, heat, or osmotic stress, linkage of the two would maximize cellular efficiency, and evidence for such a linkage exists in *Neurospora crassa* (Vitalini et al., 2007; Watanabe et al., 2007). The Neurospora circadian clock directs the rhythmic phosphorylation, and activation, of the osmotic sensing (OS)-pathway p-38-like MAPK (OS-2) (Vitalini et al., 2007). The mechanistic connection between the clock and the stress responsive MAPK pathway is unknown, and critical to understand how organisms prepare for daily predictable stresses. Furthermore, defining this link is an important step in understanding how defects in circadian clocks and defects in MAPK pathways cause similar diseases in humans, including immune system dysfunction, heart disease, neurodegenerative disorders, and cancer (Bentley et al., 2008; Cuenda and Rousseau, 2007; Fu and Lee, 2003; Han and Sun, 2007).

The OS-pathway of Neurospora is a conserved pathway similar to the HOG pathway of *Saccharomyces cerevisiae* that senses and responds to multiple environmental stresses, but is most noted for its role in the hyper-osmotic shock response (Jones et al., 2007; Posas et al., 2000; Zhang et al., 2002). Osmotic activation of this pathway ultimately results in the production of small molecules that adjust internal osmotic pressure (Banno et al., 2007; Hohmann, 2002b; Jones et al., 2007; Krantz et al., 2006; Noguchi et al., 2007; Posas et al., 2000). Input to the Neurospora OS pathway involves a phospho-relay whereby a sensor histidine kinase (OS-1) detects an environmental signal, which is propagated through a histidine phosphotransferase (HPT-1) to a response regulator (RRG-1)(Jones et al., 2007). RRG-1 modulates the activity, by an unknown mechanism,

of a MAPK cascade that includes OS-4 (MAPKKK), OS-5 (MAPKK), and the p-38-like OS-2 (MAPK) (Fujimura et al., 2003; Zhang et al., 2002). Activated MAPK regulates the activities of effector molecules, including transcription factors, other kinases, translation factors, and chromatin remodeling proteins (Bardwell, 2006; Chang and Karin, 2001; Chen et al., 2007). In Neurospora, these effectors are thought to control downstream targets that encode components needed to survive conditions of high osmolarity, as well as for conidial integrity, sexual development, and fungicide sensitivity (Angell et al., 2008; Irmler et al., 2006). The OS-2 MAPK is a homolog of the mammalian p38 family of stress activated MAPKs (SAPKs) that are activated by a variety of extracellular stimuli including UV light, heat shock, osmotic stress, or inflammatory cytokines, and the internal circadian clock (Pizzio et al., 2003). Activated p38 MAPK controls the expression of more than 100 different genes, such as those involved cell proliferation, apoptosis, and tumor suppression (Ferreiro et al., 2010; Zarubin and Han, 2005). Thus, understanding how the clock regulates the OS MAPK pathway in Neurospora will be informative for understanding this connection in higher organisms.

The circadian clock system that allows organisms to anticipate predictable changes in the environment is composed of endogenous molecular oscillators that function to generate a free-running period that is close to 24-h when the organism is kept in constant environmental conditions, and an exactly 24-h period in natural environmental cycles (Dunlap et al., 2004). The oscillators comprise the products of “clock genes” that are

organized in transcriptional-translational feedback loops (Schibler, 2006; Young and Kay, 2001). These clock genes encode transcription activators and negative elements that feedback to inhibit their own expression by disrupting the activity of the activators. Components of the oscillators receive environmental information through input pathways, allowing the oscillators to remain synchronized to the 24-h solar day (Kozma-Bognár and Kaldi, 2008; Panda et al., 2003).

Time-of-day information from the oscillator(s) is relayed through output pathways to control expression of the ccg's and overt rhythmicity. One mechanism by which the output pathways are controlled is through the rhythmic activity of transcription factors that are themselves components of the oscillator. For example, in mammals, the positive oscillator components mCLK and BMAL1 bind to E-box elements in the promoters of some clock outputs including *Dbp* and *Avp*, thereby driving rhythmic transcription (Gekakis et al., 1998; Jin et al., 1999; Muñoz et al., 2002; Ripperger et al., 2000; Yoo et al., 2005).

In the well-characterized Neurospora FRQ/WCC oscillator (FWO), the positive oscillator components WHITE COLLAR-1 (WC-1) and WC-2 dimerize to form the WCC (Talora et al., 1999). The WCC functions as a blue light photoreceptor and as a transcription factor in the core oscillator to turn on expression of *frq* encoding the negative element FREQUENCY (FRQ) (Brunner and Káldi, 2008; Cha et al., 2007; Dunlap et al., 2007; Loros et al., 2007). In addition to the role of the WCC in

photoresponses and in the oscillator, the WCC signals time of day information directly to downstream ccg's (Chen et al., 2007; He and Liu, 2005b; Smith et al., 2010; Vitalini et al., 2007). In a recent study, Smith *et al* (2010) demonstrated that the WCC binds to hundreds of genomic regions, including the promoters of previously identified clock- and light-regulated genes, as well as a suite of second tier transcription factors and signaling molecules.

We previously demonstrated that the Neurospora OS pathway functions as an output pathway from the FWO (de Paula et al., 2008; Vitalini et al., 2007). Under constant environmental conditions, time-of-day information is somehow transferred from the FWO resulting in rhythms in OS-2 phosphorylation. OS-2 phosphorylation levels peak in the early subjective morning and are at the lowest in the night. This would allow the cells to be prepared for daily daytime stress, including light, heat, and desiccation. However, the FWO is not required for Neurospora cells to mount an acute response to osmotic shock; FRQ or WC-1 deletion strains show rapid phosphorylation of OS-2 following a salt shock (Vitalini et al., 2007). This suggests that the OS pathway receives information from at least two sources: the endogenous clock and the external environment. While environmental input to MAPK pathways has been well studied (Cuadrado and Nebreda, 2010; Hohmann, 2002b), how the endogenous clock signal is perceived by the MAPK pathway is not known.

In this study, we investigated the mechanisms by which the clock regulates rhythmic

activity of the OS pathway. We found that the clock- and light-associated WCC directly regulates the MAPK pathway through rhythmic binding to the promoter of the MAPKKK gene *os-4*, resulting in rhythmic *os-4* transcription and OS-4 protein accumulation. We demonstrate that deletion of the *os-4* WCC binding sites abolishes rhythmic expression of *os-4,* disrupts rhythmic accumulation of phospho-OS-2, and abolishes circadian clock-dependent effects on the kinetics of glycerol accumulation. Additionally, a component of the phospho-relay, *hpt-1*, is shown to be clock-regulated, with mRNA levels peaking at a phase opposite of *os-4* mRNA. This anti-phase regulation of the phospho-relay and MAPK module by the clock likely contributes to the robustness of the rhythm in OS-2 activity. Whereas the major focus on the regulation of MAPK pathways has been at the level of posttranslational control of phosphorylation, our results suggest an important role for transcription initiation in the regulation of MAPK pathway components and signaling. Together, these findings may have important implications in treatments for diseases associated with defective MAPK pathways.

RESULTS

The promoter of the os-4 gene, encoding a MAPKKK, is a direct target of the WCC

To characterize the output pathways from the FWO, we previously carried out a comprehensive chromatin immunoprecipitation/sequencing (ChIP-seq) study to identify the direct targets of the WCC using antibody directed against WC-1 and WC-2 (Smith et

al., 2010). The cultures were given an 8-min light pulse prior to ChIP to activate the WCC; thus, we identified the top tier genes involved in light signaling pathways and circadian clock output pathways. Using this method, hundreds of direct targets of the WCC were identified, most of which were present in the promoters of genes, including a 500 bp region (nt 4448839-4449339 of chromosome 1) that resides about 1.7 kb upstream of the predicted start of transcription for the *os-4* gene. Within this 500 bp region of the *os-4* promoter, 3 candidate binding sites (called light-responsive elements [LRE] 1-3) for the WCC were identified that closely match a consensus binding site (GATCGA) derived from the ChIP-seq target data for the WCC (Smith et al., 2010) (Figure 2-1A).

To validate the WCC ChIP-seq results for the *os-4* promoter, an independent replicate WC-2 ChIP, followed by region-specific PCR from cultures given a light pulse was carried out (Figure 2-1B). Enrichment of WC-2 binding was observed in the same *os-4* promoter region revealed by ChIP-seq, but as expected, not in the control *cpc-1* gene which lacks a WCC binding site. Of the direct targets of the WCC, most, but not all, genes are light-induced (Smith et al., 2010). To determine if *os-4* expression is photo-induced, the level of *os-4* mRNA was measured in cells before and after a light pulse. We found that a 30-min light pulse induced *os*-4 transcript levels by about 6-fold, and that WC-1, but not RRG-1 (a component of the phospho-relay), is necessary for this induction (Figure 2-1C).

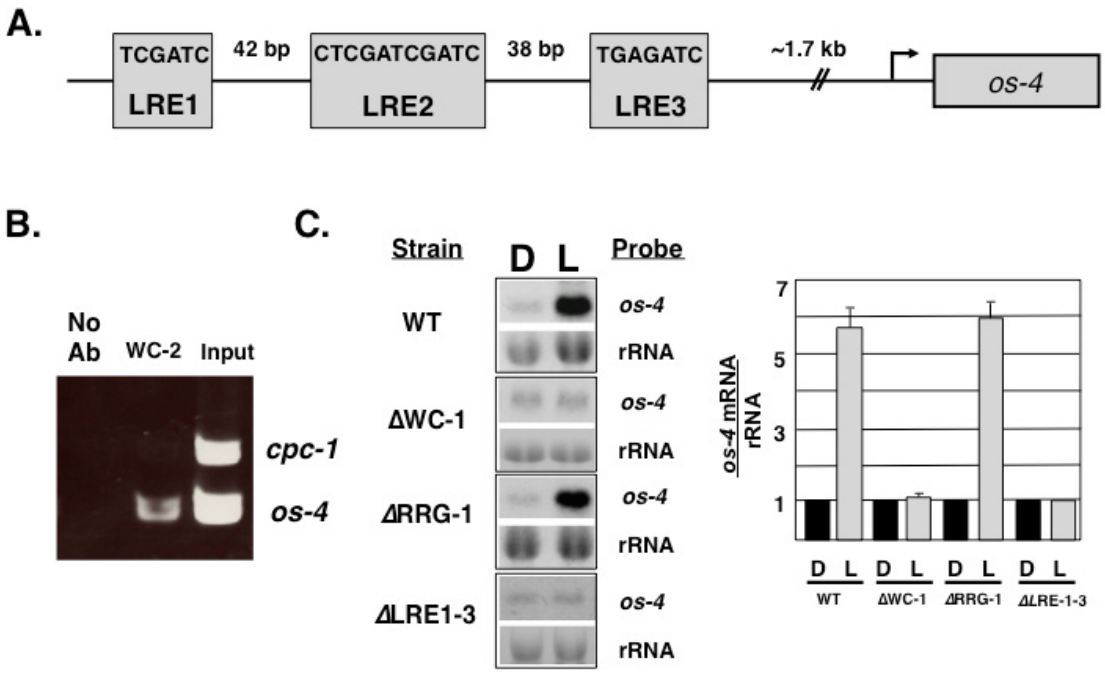


Figure 2-1. WC-2 binds to the *os-4* promoter and binding is necessary for light induction of *os-4* mRNA. **(A)** Candidate WCC binding sites within the *os-4* promoter region. The sequences of three candidate light-responsive elements (LREs) that lie within the 500 bp region of the WCC binding site, about 1.7 kb upstream of the start site of transcription are shown. The size of the drawing is not to scale. **(B)** ChIP-PCR assay using WC-2 antibody showing binding to the *os-4* promoter, but not to the *cpc-1* gene, following a 10-min light pulse of cultures. Input DNA and no antibody (no Ab) are shown as controls. **(C)** Representative northern blot analysis of *os-4* mRNA from cultures grown in the dark (D) or given a 30-min light pulse (L) in the indicated strains. rRNA is shown as a loading control. Densitometry analysis of northern blot experiments (n=4 ± SEM) is shown on the right.

Together, these data demonstrate that the WCC binds to the *os-4* promoter and activates *os-4* transcription in response to light. Consistent with this result, regulation of *os-4* by light occurs independently of a functional phospho-relay required for induction of the MAPK pathway by an acute osmotic shock (Jones et al., 2007).

The os-4 gene is regulated by the circadian clock

The WCC functions as a positive element in the FWO by binding to the *frq* promoter and activating transcription in the morning (Froehlich et al., 2002; He et al., 2002) leading to rhythms in FRQ protein. Thus, the discovery that the WCC binds to the promoter of the *os-4* gene suggested the possibility that *os-4* is expressed with a circadian rhythm. To test this idea, WT clock cells were grown in constant dark (DD) for 2 days, harvested every 4 h, and RNA isolated from the cells was probed with an *os-4*-specific probe. A robust circadian rhythm in *os-4* mRNA accumulation was observed ($p \leq 0.007$; period 21.5 ± 0.6 h), with peaks occurring in the subjective morning at 12 and 32 h in DD (Figure 2-2). As expected for a gene regulated by the FWO, this rhythm was abolished in strains deleted for either FRQ or WC-1. The levels of *os-4* transcripts were generally low in the ΔWC-1 strain, and high in the ΔFRQ strain, as compared to the WT strain. These data are consistent with direct activation of *os-4* transcription by the WCC, and regulation of *os-4* by the FWO, whereby the loss of FRQ in cells results in constitutively active WCC and high levels of *os-4* mRNA. The *os-4* mRNA rhythms persisted in an ΔRRG-1 strain (Figure 2-3), indicating that similar to the light response, RRG-1 is not

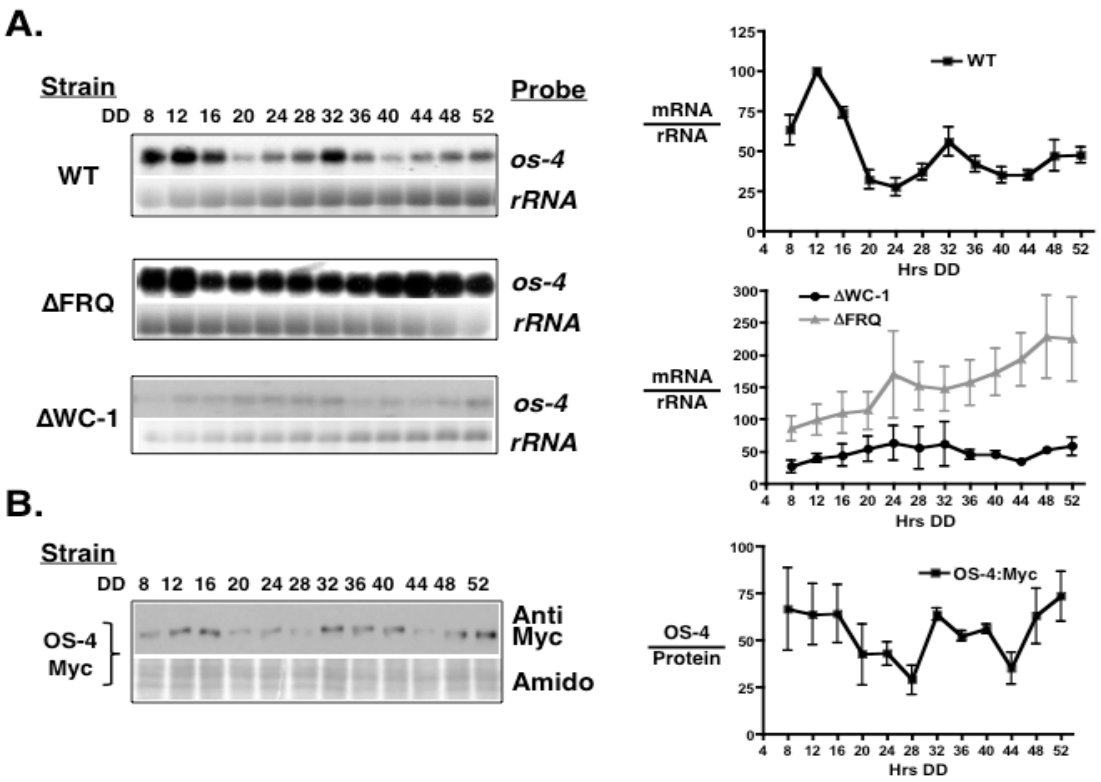


Figure 2-2. The *os-4* gene is expressed with a circadian rhythm. **(A)** Representative northern blots showing *os-4* mRNA levels in the indicated strains from cultures grown in the dark (DD) and harvested every 4-h over the course of two days (left). rRNA is shown as a loading control. Densitometric analysis of northern blot experiments are plotted and shown on the right (n=3 ± SEM). The average peak in mRNA accumulation in WT cells for each replicate experiment was set to 100. The data for ΔWC-1 and ΔFRQ are normalized to *os-4* levels in the WT strain making the levels directly comparable to each other. **(B)** Representative western blot using Myc antibody to detect the OS-4:Myc fusion protein from Neurospora cells grown as in A (left). Amido stained protein is shown below as a loading control. Densitometric analysis of western blot experiments are plotted and shown on the right (n=3 ± SEM). The average peak in protein accumulation in WT cells for each replicate experiment was set to 100.

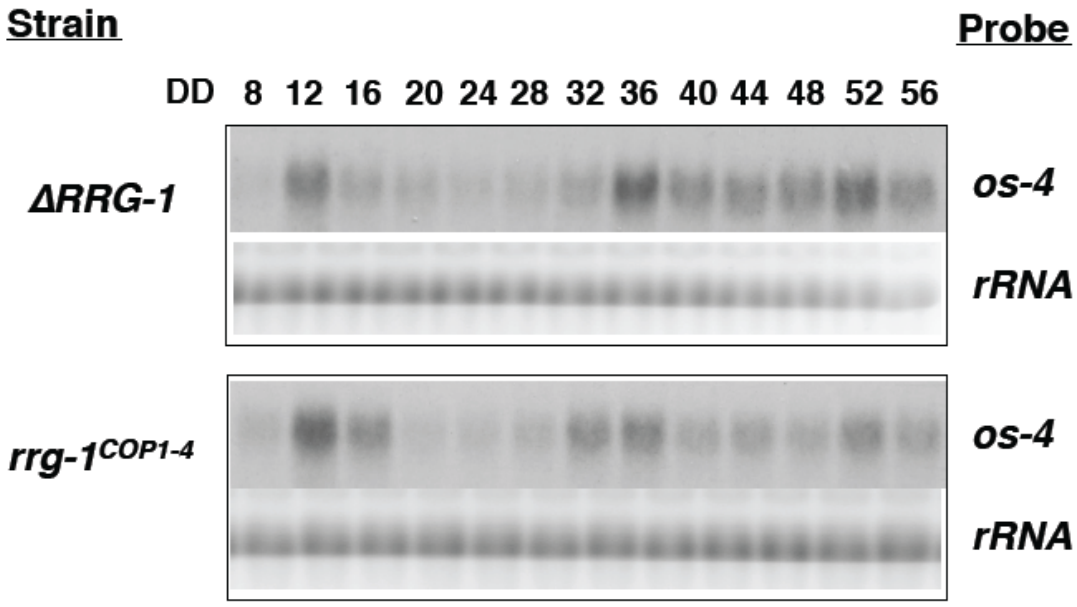


Figure 2-3. The *os-4* gene is expressed with a circadian rhythm in an *rrg-1* deletion strain (ΔRRG-1) and in an *rrg-1* null mutant strain (*rrg-1*$^{COP1-4}$). A time course experiment was carried out essentially as in Figure 2A with the strains KB1052 (*ras-1*^{bd}, Δ*rrg-1::hph*) and DBP688 (*ras-1*^{bd}, *rrg-1*$^{COP1-4}$). RNA was analyzed by northern blot. *os-4* message is shown in the top panel, and ethidium bromide stained rRNA in the bottom panel.

necessary for *os-4* mRNA rhythms. Taken together, these data suggest that clock and light input into the MAPK module occurs at the level of *os-4* transcription.

To determine if accumulation of OS-4 protein is similarly rhythmic, the endogenous *os-4* gene was replaced with an OS-4:7xMyc-tagged construct, and accumulation of the tagged protein was examined over the course of the day. The tagged protein retained normal activity, as the tagged strain was resistant to high salt conditions that are lethal to an *os-4* deletion strain (data not shown). A statistically significant, low amplitude rhythm was observed in OS-4::7xMyc protein ($p \leq 0.03$; period 20.4 ± 1.4 h), with peaks occurring in the subjective morning, similar to *os-4* mRNA peaks (Figure 2-2B).

WC-2 binds rhythmically to the os-4 promoter

We have shown that the WCC binds to the promoter of *os-4*, and that *os-4* mRNA accumulates rhythmically, peaking in the morning at the time of day when the WCC is most active (Schafmeier et al., 2005). To determine if rhythmic expression of *os-4* is due to WCC binding to the promoter at specific times of the day, WC-2 ChIP/qPCR was carried out on cultures grown in DD and harvested every 4 h over two days. For WT clock cells, an enhanced enrichment of *os-4* promoter DNA associating with the WCC in the morning samples (DD12 and DD32), and reduced enrichment in evening samples (DD24 and DD44), was observed (Figure 2-4). This is consistent with the morning-specific expression of *os-4* mRNA (Figure 2-2). In the ΔFRQ strain, *os-4* promoter DNA

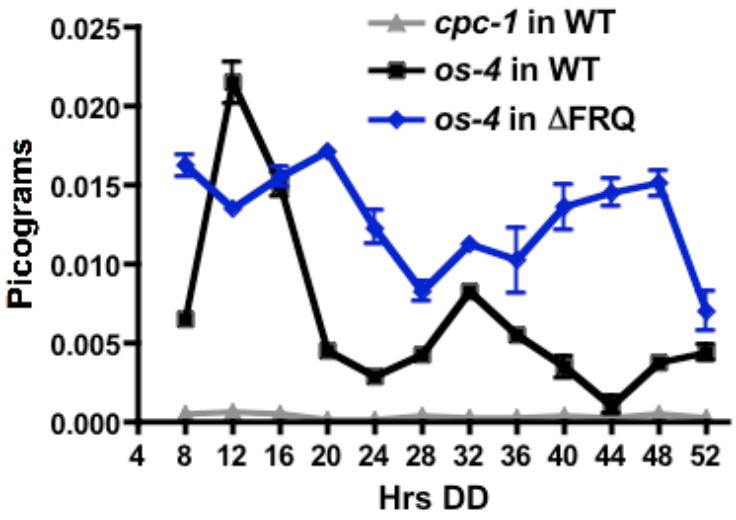


Figure 2-4. The WCC binds rhythmically to the *os-4* promoter. Plot of ChIP-quantitative PCR results for the *os-4* promoter in WT (black line and squares) and Δ*frq* (blue lines and diamonds) strains, and the control *cpc-1* gene in a WT (grey line and triangles) at the indicated times in the dark (Hrs DD). (n=4 sample replicates ± SEM). Two biological replicates yielded similar results.

associated with the WCC generally at all times of day (Figure 2-4), coincident with constitutive high levels of *os-4* mRNA in ΔFRQ cells (Figure 2-2). As a control, PCR for *cpc-1*, a gene not regulated by the WCC, was conducted. As expected, low levels of the *cpc-1* PCR product were detected at all times of day (Figure 2-4). These observations revealed that the WCC rhythmically binds to the *os-4* promoter, and that rhythmic WCC binding to the *os-4* promoter requires FRQ.

Rhythmic transcription of os-4 affects rhythmic activity of the MAPK pathway

Stress signaling through MAPK pathways results in phosphorylation of existing MAPK protein components. However, very little is known about the effects of transcriptional regulation on signaling. We have shown that the circadian clock regulates rhythmic transcriptional activation of *os-4* through rhythmic binding of the WCC to the promoter. This activity leads to rhythmic accumulation of OS-4 protein. We then asked if rhythmic transcription of *os-4* is required for rhythms in phosphorylation of the downstream p-38-like MAPK (OS-2). As OS-2 phosphorylation is undetectable in an *os-4* deletion strain (Noguchi et al., 2007), an alternate way of disrupting the rhythmicity of *os-4* expression was required. Based on the failed light induction of *os-4* in the ΔLRE1-3-*os-4* promoter mutant strain (Figure 2-1C), we predicted that this strain might also have defects in *os-4* mRNA rhythms. Figure 2-5A shows that the ΔLRE1-3-*os4* strain still maintains expression of *os-4* mRNA, but that it is not rhythmic. Thus, we tested whether the rhythms in *os-4* transcripts are required for the phosphorylation rhythms in OS-2 using

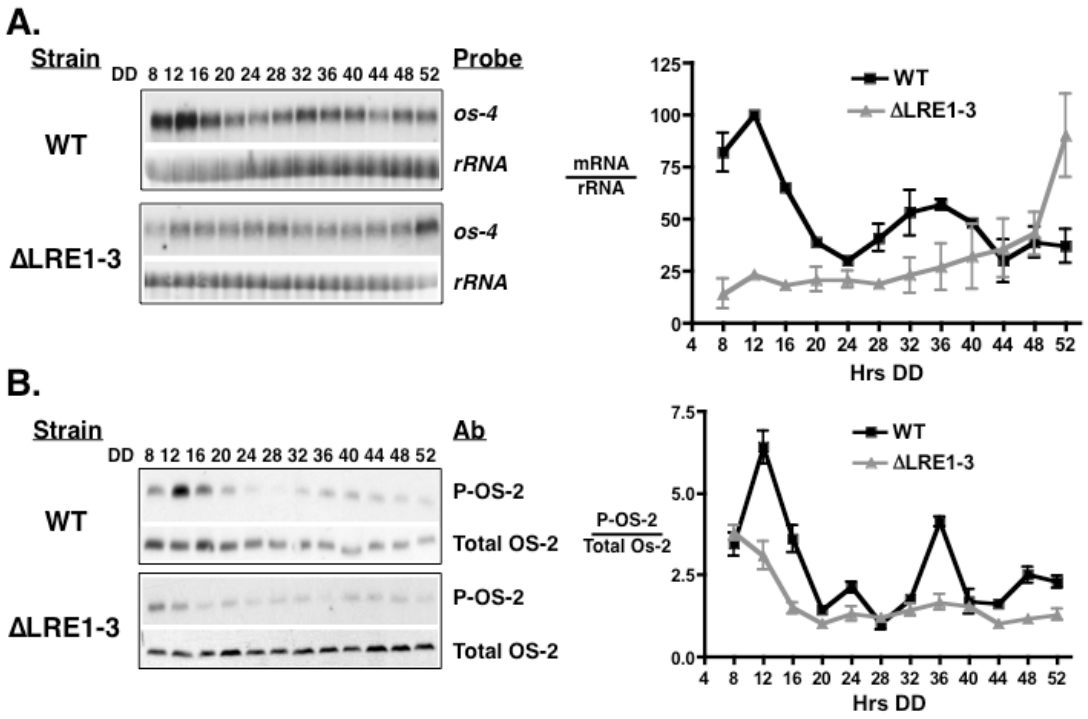


Figure 2-5. Deletion of the WCC binding site on the *os-4* promoter abolishes rhythms in *os-4* mRNA and phospho-OS-2 accumulation. (A) Northern blot assay of *os-4* mRNA from WT (top) and ΔLRE1-3-*os-4* cells (ΔLRE1-3) harvested at the indicated times in the dark (DD). rRNA serves as a loading control. Densitometric analyses of the northern blot experiments are plotted on the left as the level of *os-4* mRNA over rRNA (n=3 ± SEM). The peak levels *os-4* mRNA in WT were set to 100 for each experiment, and the data for ΔLRE1-3are normalized to the peak *os-4* mRNA levels in the WT strain making the levels directly comparable to each other. **(B)** Western blot of protein isolated from WT and ΔLRE1-3-*os-4* (ΔLRE1-3) cells and probed with phospho-specific p38 antibody (P-OS-2) or an antibody that recognized phosphorylated and un-phosphorylated OS-2 (total OS-2). Densitometric analysis of the western blot experiments are plotted as the level of P-OS-2 over total OS-2 protein, and shown on the right (n=4 ± SEM).

the ΔLRE1-3-*os-4* strain. Deletion of the LREs in the *os-4* promoter reduced the absolute levels of phospho-OS-2. Despite this reduction in phospho-OS-2 levels, the amount produced was sufficient to render the strains resistant to salt treatment (data not shown). In addition, OS-2 phosphorylation was induced by treatment of ΔLRE1-3-*os-4* cells with 4% NaCl (Figure 2-6), supporting the idea that the clock and the environment use independent pathways for activation of OS-2. In WT strains, phospho-OS-2 accumulated with a circadian rhythm ($p \leq 0.008$, period 22.8 ± 1.4 h), while deletion of the LREs in the *os-4* promoter disrupted the normal rhythms in phospho-OS-2 accumulation (Figure 2-5B). Taken together, these data demonstrate that direct activation of *os-4* transcription by the WCC in the morning is necessary for robust rhythms in OS-2 phosphorylation in constant environmental conditions.

Evening-specific regulation of hpt-1 mRNA levels by the circadian clock

To determine if the circadian clock regulates the expression of other OS pathway components, each of the pathway components was assayed for rhythmicity. No statistically significant rhythm was observed in total protein accumulation for OS-2 (Vitalini et al., 2007) (Figure 2-5), or in mRNA accumulation for the histidine kinase gene *os-1*, the response regulator gene *rrg-1*, or the MAPKK gene *os-5*, (Figure 2-7). However, a circadian rhythm was observed in the histidine phosphotransferase *hpt-1* gene mRNA ($p \leq 0.0001$, period 23.3 ± 0.8 h) and FLAG-tagged HPT-1 protein (HPT-1:FLAG) ($p \leq 0.01$, period 23.2 ± 2.1 h) (Figure 2-8A & B). The FLAG tagged HPT-1

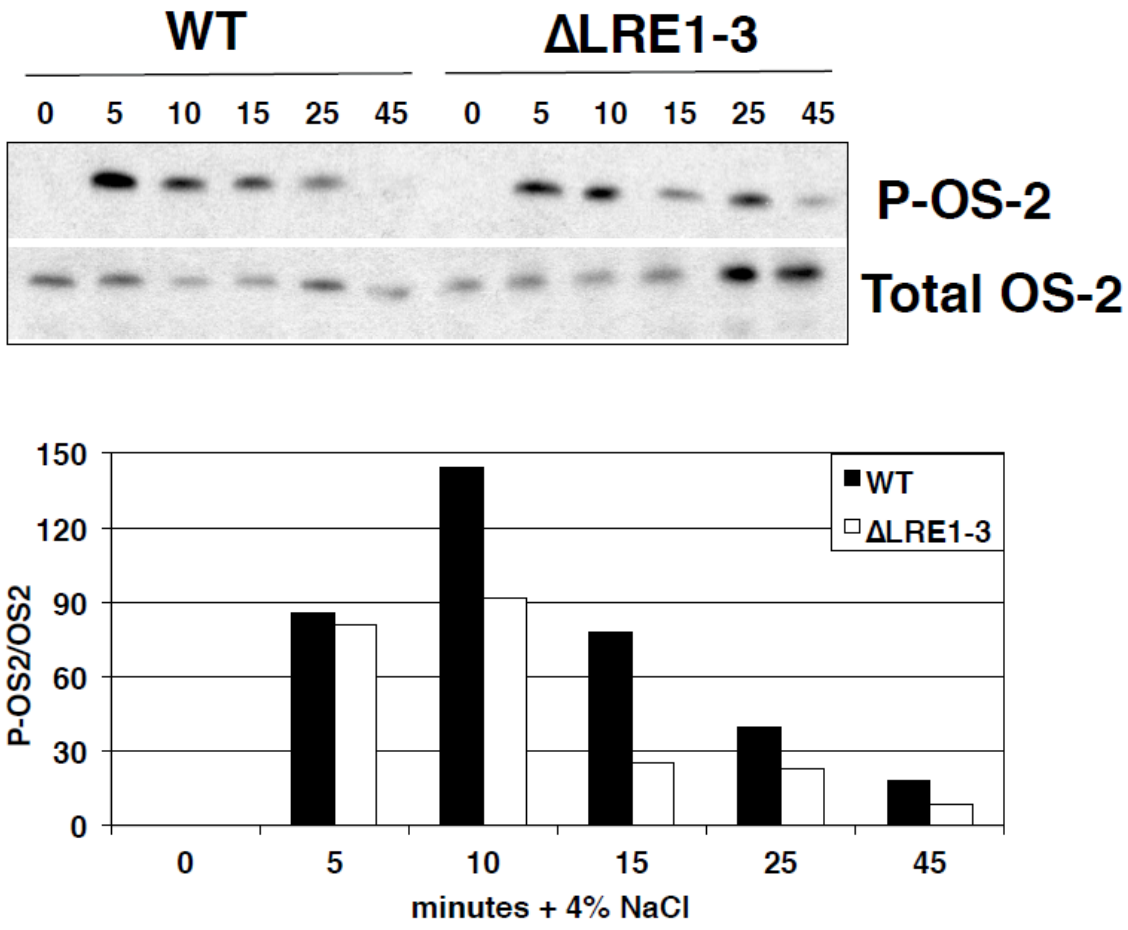


Figure 2-6. OS-2 is normally phosphorylated after salt induction in the ΔLRE1-3-*os-4* strain. Western blot of protein isolated from WT and ΔLRE1-3-*os-4* (ΔLRE1-3) cultures were subjected to a 4% NaCl treatment at DD24. Blots were probed with phospho-specific p38 antibody (P-OS-2) or an antibody that recognized phosphorylated and unphosphorylated OS-2 (total OS-2). Densitometric analysis of the western blot experiment is plotted as the level of P-OS-2 over total OS-2 protein.

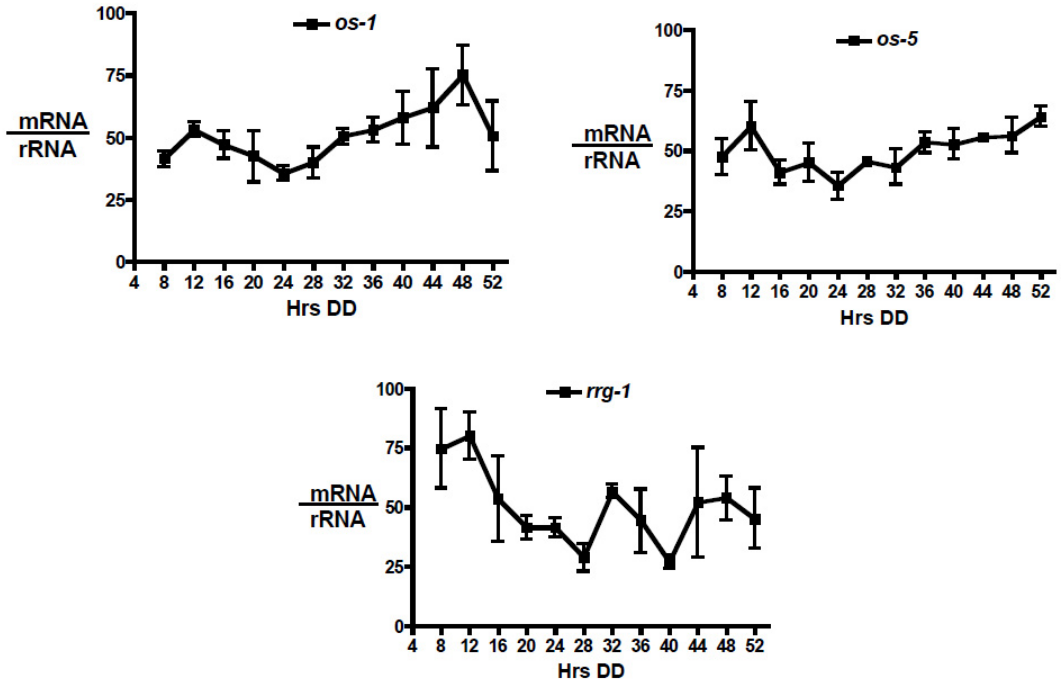


Figure 2-7: OS-pathway components *os-1*, *os-5*, and *rrg-1* are not clock regulated at the level of transcript abundance. Densitometric analysis of northern blots was carried out as described in Figure 2. The average expression level (transcript level divided by rRNA level) is plotted versus the time in DD (n=3 +/- SEM for each transcript).

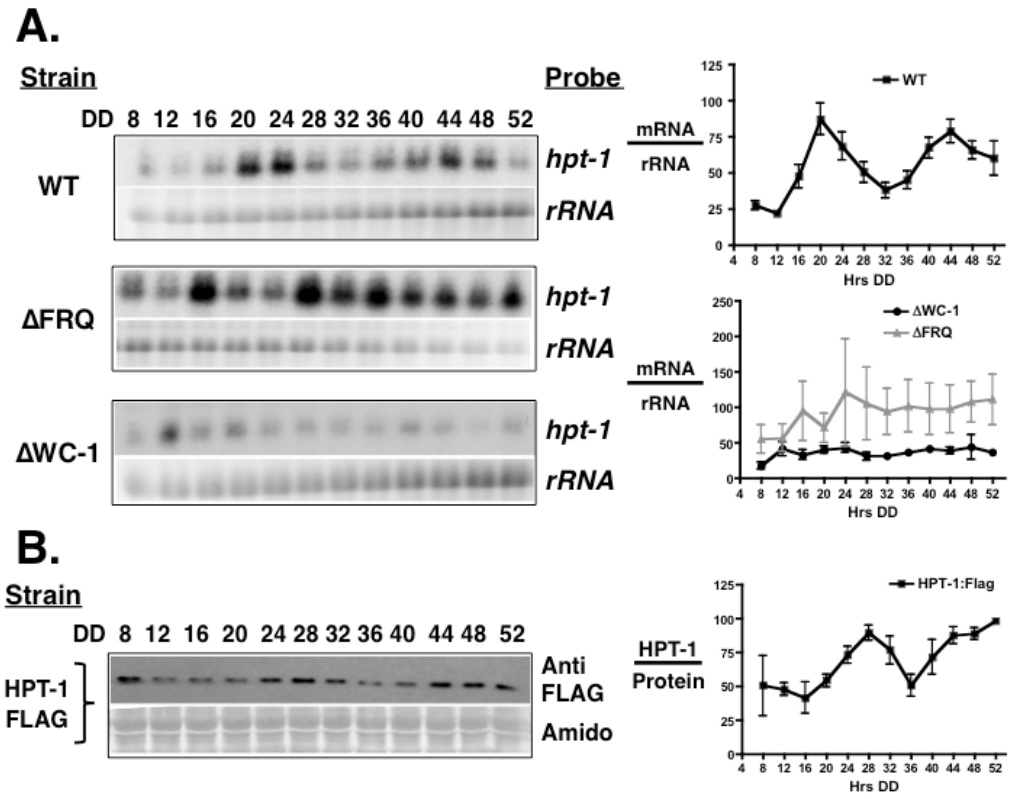
gene retained function, as a strain carrying the tag was viable (data not shown), unlike a strain carrying an *hpt-1* deletion (Banno et al., 2007).

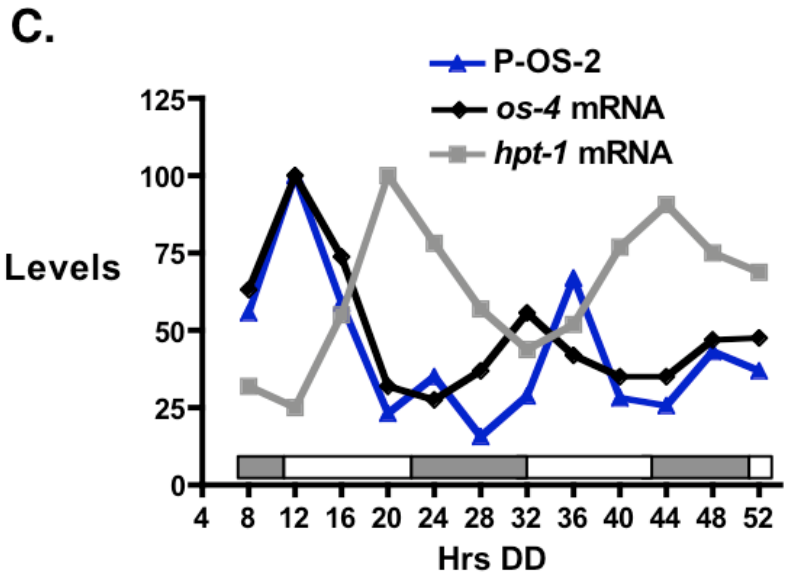
Interestingly, the peak time of accumulation for *hpt-1* mRNA is in the subjective early evening, anti-phase to the peak in *os-4* mRNA and phospho-OS-2 levels (Vitalini et al., 2007) (Figure 2-8C). As expected for a ccg, the rhythm in *hpt-1* mRNA accumulation was abolished in strains that lacked a functional FWO, with generally high levels of *hpt-1* mRNA accumulation at all times of day in the ΔFRQ strain, and low levels in the ΔWC-1 strain. Similar to *os-4,* these data are consistent with positive regulation of *hpt-1* expression by the WCC; however, this regulation is likely not direct as the *hpt-1* gene was not found in ChIP-seq to be a direct target of the WCC (Smith et al., 2010); no consensus WCC binding site is present in the *hpt-1* gene promoter, and the peak phase of *hpt-1* expression occurs when the WCC is inactive in cells grown in the dark (Schafmeier et al., 2005).

Physiological relevance of clock control of the OS pathway: kinetics of response

Activation of the OS pathway by a salt shock results in an increase in intracellular glycerol as an osmo-protectant (Noguchi et al., 2007; Zhang et al., 2002). Daily rhythms in phospho-OS-2 might be predicted to cause rhythms in glycerol levels in the absence of a stress. However, we observed no clear rhythms in glycerol levels over the course of the day. Alternatively, the levels of phospho-OS-2 observed at the circadian

Figure 2-8. The *hpt-1* gene is expressed with a circadian rhythm. **(A)** Representative northern blots showing *hpt-1* mRNA levels in the indicated strains from cultures grown in the dark (DD) and harvested every 4-h over the course of two days (left). rRNA is shown as a loading control. Densitometric analysis of northern blot experiments are plotted and shown on the right (n=3 ± SEM). The average peak in *hpt-1* mRNA accumulation in WT cells for each replicate experiment was set to 100. The data for ΔWC-1 and ΔFRQ are normalized to *hpt-1* levels in the WT strain making the levels directly comparable to each other. **(B)** Representative western blot using FLAG antibody to detect the HPT-1:FLAG fusion protein from Neurospora cells grown as in A (left). Amido stained protein is shown below as a loading control. Densitometric analysis of western blot experiments are plotted and shown on the right (n=3 ± SEM). The average peak in protein accumulation in WT cells for each replicate experiment was set to 100. **(C)** Comparison of plots of rhythmic P-OS-2 (blue line and triangles; from Figure 4B with the peak set to 100), *hpt-1* mRNA levels (grey line and squares; from Figure 5A), and *os-4* mRNA (black line and diamonds; from Figure 2A with the peak set to 100). The bars below the plot represent subjective light (white) and dark (grey).


A.
Strain
Probe
DD 8 12 16 20 24 28 32 36 40 44 48 52
WT
hpt-1
rRNA
ΔFRQ
hpt-1
rRNA
ΔWC-1
hpt-1
rRNA
mRNA
rRNA
WT
Hrs DD
mRNA
rRNA
ΔWC-1
ΔFRQ
Hrs DD
B.
Strain
DD 8 12 16 20 24 28 32 36 40 44 48 52
HPT-1
FLAG
Anti
FLAG
Amido
HPT-1
Protein
HPT-1:Flag
Hrs DD


C.
P-OS-2
os-4 mRNA
hpt-1 mRNA
Levels
Hrs DD

peak in the early morning may be lower or qualitatively different than the activation achieved by a salt shock. If this is true, we predicted that there would be a circadian difference in glycerol accumulation in response to osmotic stress. To test this possibility, WT Neurospora cells were treated at different circadian times with a salt shock, and glycerol production was monitored. As expected from our previous experiment, there was no difference in the glycerol content of presumptive morning (DD12) or presumptive evening (DD24) tissue at time 0 in any of the strains examined. After 1h in 4% NaCl, although the levels of glycerol have begun to increase, there was no time-of-treatment-dependent difference in the glycerol response for any strain (Figure 2-9). By 3 h in 4% NaCl, the WT tissue treated at DD12 showed a significant increase in glycerol content compared to the DD24 treatment. A functional clock is required for this time-of-treatment difference in glycerol, as ΔFRQ strains lack this different response. In addition, the time-of-day effect of salt treatment on glycerol levels is abolished in ΔLRE strains, suggesting that high amplitude rhythms in OS-2 phosphorylation are required for this effect. By 5 h in 4% NaCl, the acute shock had overridden any circadian control, leading to similar glycerol values when treated at DD12 or DD24.

DISCUSSION

In this study, we discovered biological regulation of a MAPK pathway by direct clock transcriptional control. The circadian clock controls expression of MAPK pathway components rather than affecting post-transcriptional signaling via receptors and

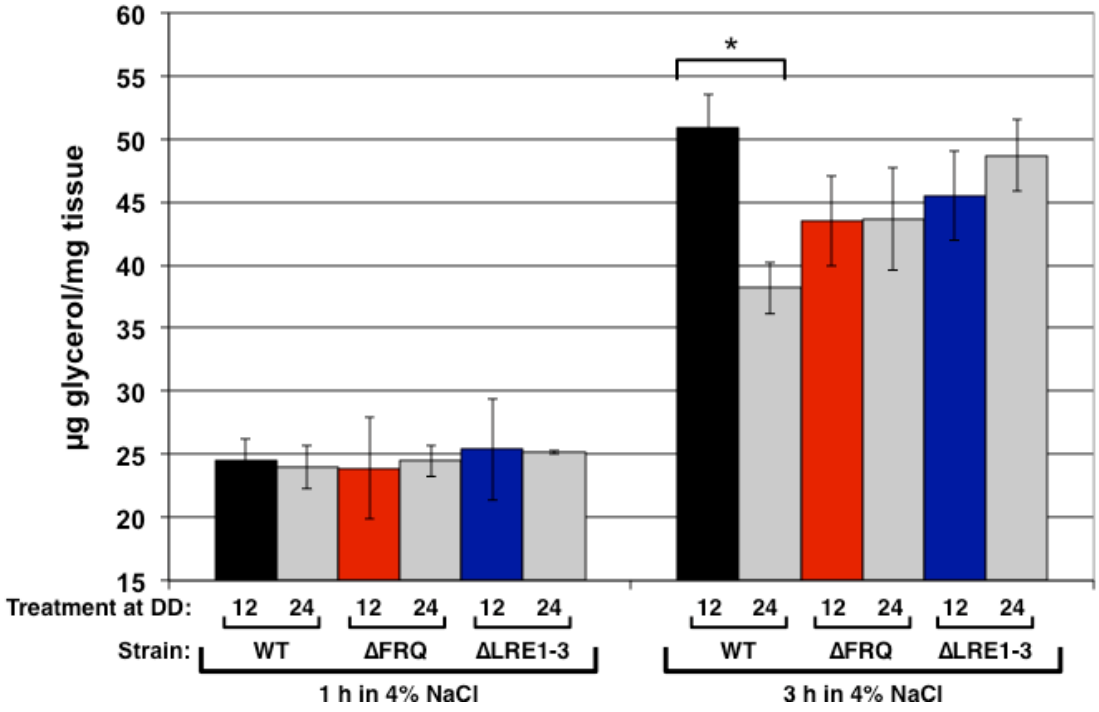


Figure 2-9. Clock-dependent kinetic advantage in glycerol accumulation following salt stress. The concentration of glycerol (µg/mg tissue) is plotted from cultures grown in constant darkness (DD) and treated with 4% NaCl for 1 or 3 hours at two different times of day (DD12 versus DD 24) in the indicated strains. A statistically significant difference in the glycerol levels was observed in WT strains treated at DD12 and DD24 ($p<0.001$; n=3, student T-test). n=3 ± SEM

phosphorylation cascades. These data suggest that circadian changes in the cellular environment, while not activating the pathway via receptor mediated stress sensation, can prime the basal activity of the MAPK pathway. As clocks and MAPK pathways are both evolutionarily conserved, and responsive to similar stressors, clock control of MAPK activation is expected to be widespread. Consistent with this idea, rhythms in the phosphorylated form of ERK, JNK, and p38 MAPKs have been observed in several higher eukaryotic model systems (Coogan and Piggins, 2003; Hasegawa and Cahill, 2004; Hayashi et al., 2001; Hayashi et al., 2003; Nakaya et al., 2003; Obrietan et al., 1998; Pizzio et al., 2003; Sanada et al., 2000; Williams et al., 2001). However, only a few examples of MAPK activity regulation via transcriptional control of the upstream MAPKKK or MAPKK are known in plants and mammals (Dóczi et al., 2007; Gallego and Virshup, 2007; Korotayev et al., 2008), and this form of regulation has not been observed in fungi. Future studies may reveal that transcriptional regulation of MAPK pathway components is more biologically relevant than thought previously.

Direct transcriptional regulation by the clock controls MAPK phosphorylation

We have demonstrated that the light responsive WCC regulates *os-4* (MAPKKK) transcription by direct promoter binding (Figure 2-1A). Not only does the WCC bind and induce *os-4* transcript in response to a light pulse, but also in DD the WCC rhythmically binds to the *os-4* promoter to drive rhythmic transcription (Figures 2-2 & 2-3). Three prospective WCC binding sites in the *os-4* promoter were identified (Figure 2-1C) and

deleted *(*ΔLRE1-3-*os-4*) to test their role in light responses and circadian rhythm generation. This deletion rendered *os-4* expression unresponsive to a light pulse (Figure 2-1B). Furthermore, consistent with the function of WCC as the positive circadian element, the triple binding site mutant also eliminated the circadian rhythms in *os-4* mRNA (Figure 2-5A). Expression of *os-4* was reduced, but not abolished, suggesting that other promoter elements and transcription factors are responsible for basal transcription. We do not know whether the three binding sites play differential roles in light or circadian regulation like the proximal and distal LREs in the *frq* promoter (Froehlich et al., 2002; Froehlich et al., 2003b), but future studies can address that question. Finally, and most significantly, the triple WCC binding site deletion of *os-4* disrupted the rhythm in phosphorylated OS-2 MAPK (Figure 2-5B) and circadian regulation of glycerol induction kinetics (Figure 2-9). These observations support our conclusion that transcriptional control of the MAPK pathway components leads to rhythms in MAPK pathway sensitivity.

Elucidating the mechanism of day/night regulation of the OS-MAPK pathway

We have demonstrated that the FWO regulates transcript and protein levels of *os-4* and *hpt-1* (Figures 2-2 & 2-8). *os-4* is a direct clock target peaking in the subjective morning/day, and *hpt-1* is an indirect target peaking in the subjective evening/night. Our model (Figure 2-10) proposes that by controlling levels of OS-4 and HPT-1 proteins, the clock can tune the basal level of OS pathway activation in the absence of an osmotic

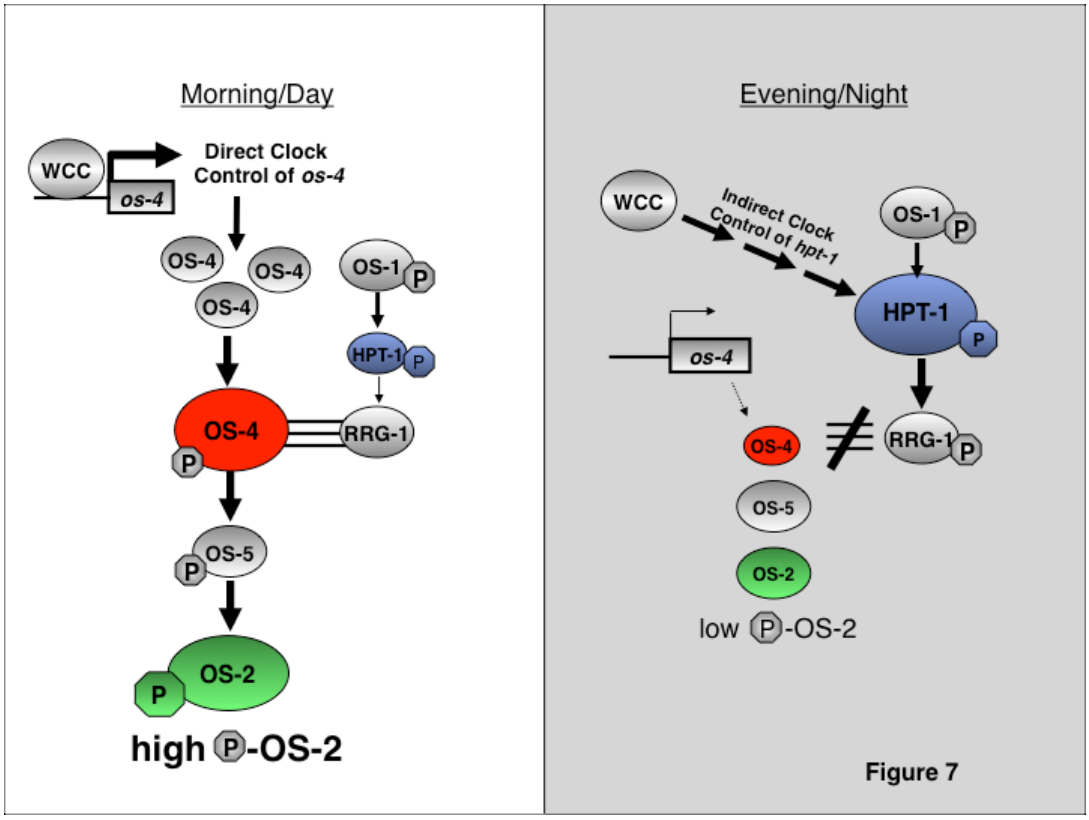


Figure 2-10. Model of circadian regulation of the OS MAPK pathway. See the text for details of the model. Single arrows refer to direct control and multiple arrows indicate indirect control. P= phosphorylation. Triple lines indicate physical interaction. The sizes of the circles for OS-4, HPT-1, and P-OS-2 reflect their relative levels during the day versus the night.

shock. OS-4 is a positive regulator of phospho-OS-2, while HPT-1 is a predicted negative regulator of phospho-OS-2 through phosphorylation of RRG-1 (Fujimura et al., 2003; Jones et al., 2007). Because these two genes are expected to have opposite effects on the regulation of OS-2 activity, their anti-phase expression rhythms should synergize to activate OS-2 in the morning/day, and inactivate OS-2 in the evening/night (Figure 2-10). For instance, in the subjective morning, at DD12, OS-4 protein is at a peak, and low HPT-1 levels reduce the levels of RRG-1 phosphorylation, which is thought to increase RRG-1 interaction with OS-4 and increase MAPK activation. Both the decrease of HPT-1 and increase of OS-4 protein levels would promote the phosphorylation of OS-2. Conversely, during the subjective night, at DD24, OS-4 is down-regulated while HPT-1 is up-regulated. An increase is HPT-1 would produce more phosphorylated RRG-1. Based on similarities to the yeast HOG pathway (Hohmann, 2002a), phosphorylated RRG-1 is predicted to not physically interact with OS-4; thus, reducing MAPK pathway activation. At the same time, OS-4 protein is low. Therefore, in the night the clock discourages phosphorylation of OS-2 by increasing HPT-1 levels and decreasing OS-4 levels. Consequently, the dual clock inputs to the MAPK pathway work together to strengthen the day/night variation in MAPK activity.

Even though both inputs to the MAPK pathway are thought to contribute to the robustness of the rhythm in MAPK phosphorylation, disruption of just the direct input to *os-4* (ΔLRE1-3-*os-4* mutant) is sufficient to abolish the high amplitude phosphorylation rhythm in OS-2 (Figure 2-5) and the circadian regulation of glycerol induction kinetics

in response to stress (Figure 2-9). This suggests a dominant role for rhythmic MAPKKK in generating rhythms in MAPK sensitivity. Evening-specific regulation of *hpt-1* could be achieved through indirect regulation from the WCC using a linear series of transcriptional activators (*i.e.* a morning-specific activator that is a direct target of the WCC activates another transcription factor that peaks later in the day, etc.), or through activation of a repressor by the WCC. However, our data support the first alternative; the levels of *hpt*-1 mRNA are low in the ΔWC-1 strain as compared to the WT strain, suggesting positive regulation of *hpt-1* by the clock. Experiments are currently underway to identify the transcription factor(s) that regulate *hpt-1* evening-specific transcription. Identification of this transcription factor(s) responsible for *hpt-1* mRNA rhythms will allow us to disrupt the binding site to determine if the rhythm in *os-4* transcription is sufficient for phospho-OS-2 rhythms, and if the rhythm in *hpt-1* contributes to the robustness of the phospho-OS-2 rhythm.

Physiological consequences of the OS-2 phosphorylation rhythm

Dual threonine/tyrosine phosphorylation of a MAPK activates the kinase domain and brings about the full complement of downstream effects. We initially identified the OS-MAPK pathway as controlling the circadian expression of *ccg-1* (Vitalini et al., 2007), and one reasonable hypothesis would be that genes controlled by this pathway in response to osmotic stress might also be circadianly regulated. While there are other stress-induced and circadian outputs of this pathway (ex. *ccg-9*, trehalose synthase),

several OS-pathway-induced genes do not show rhythmic expression (TML and DBP, unpublished data). Furthermore, glycerol levels were constant in tissue harvested over the course of the day, suggesting the possibility that the dual phosphorylated OS-2 MAPK in circadian time courses is not fully active. In other words, there may be qualitative or quantitative differences in the activity of the MAPK protein resulting from how the pathway is activated. Such differences may arise from modified MAPK protein interactions, feedback mechanisms, or through other factors controlling the activity of the MAPK in clock-driven versus acute osmotic activation of the pathway. The fact that there is a different kinetic response in glycerol production at the subjective morning compared to subjective night when Neurospora is treated with an osmotic stress (Figure 2-9), suggests that the circadian effect is one of priming the pathway, not necessarily fully activating the pathway. However, in nature, environmental and clock inputs converge on signal transduction pathways simultaneously. The integration of transcriptional control and acute activation of signal transduction pathways likely coordinate to maximally activate the OS-pathway in the morning/day when osmotic stress is most damaging.

While the mechanism of the circadian oscillator transcriptional negative feedback loop is highly conserved within the eukaryotes, the core oscillator components and/or their roles in the oscillator vary among phyla (Loros, 1998; Rosato and Kyriacou, 2001). In contrast, the MAPK pathway components (Widmann et al., 1999), and clock-associated kinases and phosphatases (Gallego and Virshup, 2007), are highly conserved within

eukaryotes. These observations suggest that circadian clocks evolved in the context of existing MAPK signaling pathway kinases, and co-opted these pathways to control rhythmicity in target genes of the pathways that are already geared to respond to environmental signals that recur with a 24 h periodicity. This makes sense, as it would provide a simple mechanism to coordinately control sets of genes that allow the organism to anticipate and respond to the daily occurrence of a particular event. Furthermore, in view of the connection between the circadian clock and MAPK pathways, it is probably not coincidental that defects in the clock and in MAPK signaling pathways share many commonalities in human disease, including immune system defects, cardiovascular disease, and cancer (Cuenda and Rousseau, 2007; de Paula et al., 2008; Fu and Lee, 2003; Gery and Koeffler, 2007; Han and Sun, 2007). Thus a complete understanding of how the circadian clock and MAPK pathways are integrated in cells, including the role of transcriptional regulation of MAPK components by the clock, is essential to begin to solve these important issues in human health.

MATERIALS AND METHODS

Strains and growth conditions

Media for vegetative growth conditions and crossing protocols are described (Davis, 1970). All strains contained the *ras-1*^{bd} mutation, which clarifies the developmental rhythm on long growth tubes. For simplicity, these are referred to as wild type (WT)

with respect to the clock throughout. Strains containing the *hph* cassette were maintained on Vogels minimal media supplemented with 200 µg/mL of hygromycin B (Calbiochem, Darmstadt, Germany). Stains containing the *bar* cassette were maintained on Vogels minimal media lacking NH_4NO_3 and supplemented with 0.5% proline and 200 µg/mL BASTA (Bayer). Time course experiments were conducted as described (Correa et al., 2003) with the following modifications: Media (1X Vogels salts, 0.5% arginine, 2% glucose, pH 6.0), synchronization (30°C lights on to 25°C dark (DD)), and shifting scheme. Liquid shaking cultures of mycelia were grown in constant light (LL) for a minimum of 4 h and transferred to DD on day 1 [for collection at DD 36, 40, 44, 48, 52], day 2 [for collection at DD 12, 16, 20, 24, 28, 32], day 3 [for collection at DD8], and harvested either at 9:00 a.m. (DD 12, 16, 20, 36, 40, 44) or 5:00 p.m. (DD 8, 24, 28, 32, 48, 52) on day 3. Tissue for RNA, protein, or ChIP analysis was harvested by flash freezing in liquid N_2 at the indicated times in DD for each experiment.

Strain construction

The ΔLRE1-3-*os-4* strain (DBP 1276; *mat a, ras-1*^{bd}*, os-4*$^{\Delta LRE1\text{-}3}$) was obtained as a homokaryon by crossing the heterokaryon (*mat a, os-4*$^{\Delta LRE1\text{-}3}$*,* Δ*wc-1::bar*$^{+}$*,* Δ*mus52::hph*) with FGSC 2489 (74-OR23-IV, *mat*A) to generate DBP 1245 (*mat a, os-4*$^{\Delta LRE1\text{-}3}$). DBP 1245 was then crossed to FGSC 1858 (*mat A, ras-1*^{bd}) to incorporate *ras-1*^{bd}. The heterokaryotic parent was obtained by co-transforming FGSC 9568 (*mat a,* Δ*mus52::hph*) with a Δ*wc-1::bar*$^{+}$ deletion construct and an unmarked ΔLRE1-3

deletion construct (consisting of the genomic region found in LGI supercontig 1 nt 4450161-4447943, with the three LREs between nt 4449236-4449133 deleted). Proper integration of the ΔLRE1-3-*os-4* construct at the native locus was confirmed by PCR and sequencing. The OS-4::MYC strain (DBP 1176; *mat A, ras-1^{bd}, OS-4::7xMYC, his-3^{+}::bar^{+}*) was obtained as a homokaryon after crossing DBP 1074 (*mat A, ras-1^{bd}, OS-4::7xMYC, his-3^{+}::bar^{+},* Δ*mus52::hph*) with FGSC 1859 (*mat a, ras-1^{bd}*). DBP 1074 was obtained by co-transforming DBP 636 (*mat A, ras-1^{bd}, his-3,* Δ*mus52::hph*) with pDBP409 (C-terminal MYC-tagged OS-4 construct, see below) and pBM61-bar^{+}. Proper integration of the MYC-tagged OS-4 construct at the native locus was confirmed by PCR and sequencing confirmed that no mutations were introduced. The HPT-1::FLAG strain DBP 1167 (*mat a, ras-1^{bd}, HPT-1::3xFLAG::hph*) was obtained as a homokaryon after crossing the heterokaryon DBP1072 (*mat a, HPT-1::3xFLAG::hph,* Δ*mus52::bar^{+})* with FGSC 1858 (*mat A, ras-1^{bd}*). DBP1072 was obtained by transforming FGSC9719 (*mat a,* Δ*mus52::bar^{+}*) with pDBP396 (C-terminally FLAG-tagged HPT-1 construct, see below). Integration of the FLAG-tagged HPT-1 construct was confirmed by detection of a ~20 kDa FLAG tagged protein (HPT-1::FLAG predicted size= 19.4 kDa) in the transformants by western blot.

Plasmid construction

Plasmid pDBP409 contains 2.4 kb of the C-terminal end of *os-4* (ending one codon before the stop codon) linked in frame to a 7xMYC tag obtained from pMF276 (Honda

and Selker, 2009) followed by 2.0 kb of the 3' UTR of *os-4*. Sequencing of the plasmid insert revealed that the hybrid PCR deleted some of the MYC tags (7x MYC instead of 13x MYC), but no other mutations were observed. Plasmid pDBP396 has a pRS426 (high copy yeast URA3 plasmid) backbone carrying the following insert; 658 bp of the C-terminal end of *hpt-1* (ending one codon before the stop codon), an in frame 17x glycine linker, an in frame 3xFLAG-tag, the hygromycin B resistance gene, *hph*, flanked by *LoxP* sites, followed by 796 bp of the 3'UTR of *hpt-1*. Sequencing of the plasmid insert determined that recombination in yeast was uneven yielding extra glycines (17x Gly instead of 10x Gly), however, no other mutations were found.

Nucleic acid isolation, hybridization, and sequencing

RNA was prepared as described (Bell-Pedersen et al., 1996) and transcripts were detected in Northern blots using a [α-^{32}P]-UTP labeled anti-sense RNA probe for *os-4*, and a [α-^{32}P]-dCTP labeled DNA probe for *hpt-1* (Correa et al., 2003).

Protein isolation and western blotting

To assay levels of OS-2 phosphorylation, protein was extracted as described (Jones et al., 2007) with the following modification: The extraction buffer used was 100mM Tris pH 7.0, 1% SDS, 10 mM NaF, 1 mM PMSF, 1 mM sodium ortho-Vanadate, 1X HALT Protease Inhibitor Cocktail (Thermo Scientific, Waltham MA). Protein concentration

was determined using NanoDrop spectroscopy (A_{280} of 1= 1mg/ml protein), and 50 µg of protein were boiled for 5 minutes in 1X Laemmli sample buffer. Samples were run on 10% SDS/PAGE gels and blotted to an Immobilon-P nitrocellulose membrane (Millipore, Billerica MA) according to standard methods. Phospho-OS-2 was detected by western blot using Mouse anti-phospho p38 primary (#9216 Cell Signaling, Beverley MA), and anti-Mouse-HRP secondary (#170-6516 BioRad, Hercules, CA) antibodies. Total OS-2 protein was detected by western blot with Rabbit anti-Hog1 primary (sc-9079 Santa Cruz Biotech, Santa Cruz, CA), and anti-Rabbit HRP secondary (#170-6515 BioRad, Hercules CA) antibodies. Immuno-reactivity was visualized on X-ray film (Phenix, Candler, NC) with Super Signal West Pico chemi-luminescence Detection (Thermo Scientific, Waltham, MA).

For detection of OS-4::MYC and HPT-1::FLAG proteins, extracts were prepared in buffer (50 mM HEPES pH 7.4, 137 mM KCl, 10% glycerol, 5 mM EDTA) (Garceau et al., 1997), supplemented with 1X HALT protease inhibitors (Thermo Scientific, Waltham, MA), and phosphatase inhibitors: 20 mM β-glycerophosphate, 5 mM Sodium Flouride,1 mM Sodium Vanadate. For OS-4::MYC, 50 µg of total proteins were run on a 6% SDS-polyacrylamide gel, and the tagged protein detected by western blot using 9E10 Mouse anti-c-MYC primary (SC-40, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Mouse-HRP secondary (#170-6516, BioRad, Hercules, CA) antibodies. For HPT-1::FLAG, 50µg of total proteins were run on a 12% SDS-polyacrylamide gel, and the tagged protein detected by western blot using Rabbit anti-FLAG primary (#2368, Cell

Signaling Technology, Beverley, MA), and anti-Rabbit-HRP secondary (#170-6515, BioRad, Hercules, CA) antibodies. Detection for both proteins was by chemi-luminescence using Super Signal Pico detection (Thermo Scientific, Waltham MA).

WC-2 ChIP

Chromatin immunoprecipitation (ChIP) was performed as described in (He and Liu, 2005a; Johnson et al., 2002) with the following modifications. Dark grown liquid shake cultures were cross-linked in 1% (v/v) formaldehyde (Sigma, St. Louis, MO) for 30 min, and then quenched with 125 mM Glycine for 5 min. Tissue was then rinsed for 5 min in 1X TBS, excess liquid removed on paper towels, and then frozen in liquid N_2. Tissue was crushed with mortar and pestle under liquid N_2 and approximately 0.5 ml ground tissue was suspended in 1 ml lysis buffer (50mM HEPES pH 7.5; 137mM NaCl; 1mM EDTA; 1% Triton X-100; 0.1% Na deoxycholate; 0.1% SDS; 1X HALT protease inhibitor cocktail). DNA was fragmented to an average size of 500-1000 bp by probe sonication (Branson Sonifier, microtip probe). Extracts were clarified by 10 min centrifugation at 14K rpm, and a Bradford assay was performed on the supernatant. The protein concentration of all extracts was normalized to 2 mg/mL with lysis buffer. Extract (1 ml) was incubated with 2 µl anti-WC-2 antisera (a generous gift from Y. Liu) with gentle shaking overnight at 4°C. Protein G/agarose beads (50 µl) (GE Healthcare, UK) blocked with salmon sperm DNA and BSA were added and incubated at 4°C for 6 h with end over end rotation. Beads were successively washed for 5 min with low salt IC buffer (0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris pH 8; 150mM NaCl),

high salt IC buffer (0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris pH 8; 500mM NaCl), LNDET buffer (0.25M LiCl; 1% NP-40; 1% Na deoxycholate; 1mM EDTA; 10mM Tris pH 8), and twice with 1X TE buffer. To elute bound protein complexes, beads were suspended in 250 µl elution buffer (0.1M NaHCO3; 1% SDS) and heated to 65°C for 15 min with periodic mixing. The supernatants from two elutions were pooled (500 µl total) and cross-links were reversed by the addition of 20 µl 5M NaCl and incubation at 65°C for ≥6 h. To degrade residual protein, samples were incubated at 50°C for 1 h with 40 µg/ml of proteinase K. DNA was isolated by phenol/chloroform/iso-amyl alcohol extraction followed by ethanol precipitation using 20 µg of glycogen as a carrier. DNA was resuspended in 50 µl 1X TE and subsequently analyzed by quantitative or semi-quantitative PCR.

Analysis of ChIP

Semi-quantitative PCR was used to examine the light-induced binding of the WCC to the *os-4* promoter. Primers used to detect genomic regions present in the immunoprecipitated DNA were as follows: for *os-4* (os4F 5'-AACCTGGTCAGAACG-CATCATA-3' and os4R 5'- GCCGGAAATGAGATGACGAA-3'); for *hpt-1* (hpt1F 5'-CTGTGCGAGTTCCTCCATGCCG-3' and hpt1R 5'- GATGAGGCAACACAGCCTT-GACG-3'); for *cpc-1* (cpc1F 5'-AAAACCCAGACACGTGGTTCTC'3 and cpc1R 5'-GGAGGGCTGACAGACGACTTC-3'). Up to two primers sets were multiplexed in a single PCR reaction. PCR cycles were as follows: 95°C for 2 min; 95°C for 15 sec.;

60°C for 30 sec.; 72°C for 30 sec. (step 2-4 repeated 25x); 72°C for 7 min. PCR products were separated on 8% acrylamide gels and visualized by ethidium bromide fluorescence under UV light.

Immunoprecipitated DNA from a rhythmic time series was analyzed by absolute quantitative PCR using Fast SYBR Green Mastermix and Fast 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). For each of the primer sets listed above, a standard to be used for absolute quantification was generated by amplifying a PCR product from genomic DNA that includes the genomic region to be analyzed. Primers used to generate standards were as follows: *os-4* (os4conF 5'-GACAATACGTCCTCTG-CAGGATGTG-3' and os4conR 5'-CTGACCTGCAGCATCGTGAC-3'); *hpt-1* (hpt1conF 5'-CCAGTTGAGTCAGATCGTTCAGGTG-3' and hpt1conR 5'-CGTGCG-TCTGATTCGCAGAAC-3'); *cpc-1* (cpc1conF 5'-CTCTACAGAATAGCGCGCGCAT-C-3' and cpc1conR 5'-CGTGCGTCTGATTCGCAGAAC-3'). The standard PCR product was purified using the PCR Clean-up Kit (Qiagen, Valencia, CA) and quantitated using gel electrophoresis and ethidium bromide staining compared to a DNA sample of known concentration. The concentration of the standard was determined by densitometric comparison between the two samples.

Glycerol assay

Neurospora tissue was ground under liquid N_2 and assayed for glycerol content using the

Free Glycerol Reagent (Sigma, F6428) and Glycerol Standard Solution (Sigma G7793) protocol supplied by Sigma.

Statistical analysis

Nonlinear regression to fit the rhythmic data to a sine wave (fitting period, phase, and amplitude) and a line (fitting slope and intercept), as well as Akaike's information criteria tests to compare the fit of each data set to the 2 equations, were carried out using the Prism software package (GraphPad Software, San Diego, CA). The p values reflect the probability that, for instance, the sine wave fits the data better than a straight line. The student T-test was used to determine significance in the difference in levels of glycerol from 3 independent experiments. The error bars in all graphs represent SEM from at least 3 independent biological replicates.

CHAPTER III

P38 MAPK IS RHYTHMICALLY ACTIVATED BY THE CIRCADIAN CLOCK IN IMMORTALIZED CELL LINES DERIVED FROM MOUSE FIBROBLASTS AND THE SUPRACHIASMATIC NUCLEUS

INTRODUCTION

To cope with daily changes imposed by the earth's rotation, diverse eukaryotic organisms have developed the ability to sense time (Bell-Pedersen et al., 2005; Brunner and Schafmeier, 2006; Dibner et al., 2010; Hardin, 2005). This internal timing mechanism, called the circadian clock, cycles with a period of about a day and provides a mechanism that allows organisms to coordinate certain aspects of their behavior or physiology to the time of day that is most advantageous (Woelfle et al., 2004). For instance, the immune system of flies and plants is regulated by the circadian clock, peaking at a time of day in which infections are most likely to occur (Dodd et al., 2005; Lee and Edery, 2008), and a functional clock is important for animals in the wild such that their activity occurs out of phase with the activity of major predators (DeCoursey and Krulas, 1998). Additionally, circadian clocks have a broad influence on human health, from cancer (Schernhammer et al., 2001) to rheumatoid arthritis (Straub and

Cutolo, 2007).

The proper functioning of a circadian clock requires several components that help the organism exist in harmony with its environment (Dibner et al., 2010). At the core of the clock is a molecular oscillator that can keep time even in the absence of environmental temporal cues (Dunlap et al., 1999). In mammals, the molecular oscillator functions as a negative feedback loop where the gene products of the positive elements (Clock/Bmal1) activate expression of negative components (Per and Cry genes) which, in turn, repress the activity of the positive elements (Mohawk et al., 2012). This molecular oscillator takes approximately 24 hrs to complete one cycle, thereby giving an organism an accurate estimate of environmental time. However, to ensure proper synchrony with the environment, the molecular oscillator is coupled to environmental sensing input pathways, with light being the strongest entrainment signal to reset the oscillator (Dibner et al., 2010). In mammals, light is perceived by the retina, which sends a neuronal signal to a region of the brain called the SCN (Dibner et al., 2010). The SCN is primarily responsible for synchronizing cellular oscillators throughout the organism to the appropriate time of day (Yoo et al., 2004). Finally, through output pathways, the oscillator orchestrates overt rhythmic processes in the organism by regulating the expression of target genes (Doherty and Kay, 2010). Although components of the molecular oscillator can directly regulate the expression of ccg's, many of these clock regulated genes are themselves able to regulate gene expression, such as transcription factors (Koike et al., 2012; Smith et al., 2010). Another way that the circadian clock

controls gene expression is through the regulation of established signaling pathways (de Paula et al., 2008).

Intracellular signaling pathways act as a biochemical means to relay information within the cell. The MAPK is a signaling pathway that operates through a successive cascade of three kinases to phosphorylate target proteins (Roux and Blenis, 2004). Upon activation of the pathway, the most upstream kinase, called a MAPKKK, phosphorylates its cognate MAPKK, which leads to activation of its kinase activity. The MAPKK phosphorylates the terminal MAPK protein, which, when active can phosphorylate targets such as transcription factors and other downstream regulatory kinases (Roux and Blenis, 2004). MAPK signaling pathways are ubiquitous in eukaryotes, and organisms commonly have multiple MAPK pathways that operate in parallel. In mammals, three conserved MAPK subfamilies have been established: ERK, JNK, and p38 (Johnson and Lapadat, 2002). These parallel MAPK pathways have varied functions depending on cell and tissue type.

Many of the mammalian MAPK pathway components participate in the functioning of the circadian clock. ERK MAPK activity cycles in the SCN of mice kept in constant conditions, presumably acting as an output pathway of the circadian oscillator (Obrietan et al., 1998; Pizzio et al., 2003), as well as playing an important role in light input to the oscillator (Akashi and Nishida, 2000; Cermakian et al., 2002; Obrietan et al., 1998). Accordingly, in model organisms such as the fungus Neurospora, ERK MAPK has a

well-established role in circadian output to regulate the expression of clock controlled genes (Bennett et al., 2013). Similarly, in Drosophila, rhythms of ERK MAPK activity are necessary for behavioral rhythms (Williams et al., 2001). In addition, JNK MAPK functions in mammals as an input to the clock in the SCN and in peripheral tissues (Yoshitane et al., 2012).

A third type of MAPK pathway, the p38 MAPK pathway, functions in circadian input in the chick pineal gland (Hayashi et al., 2003), and displays a circadian oscillation in activity in the hamster SCN (Pizzio et al., 2003). While most of the evidence for p38 as a circadian signal has focused on its role as a clock input in neural tissues, little is known about the role p38 plays in peripheral tissues, nor its likely activity in circadian output pathways. Work on the homologous OS-2 MAPK in Neurospora has shown that the p38 MAPK is rhythmically activated and functions in output pathways of the circadian clock to prepare the organism for daily changes in osmotic stress (Lamb et al., 2011; Vitalini et al., 2007). In animal systems, the p38 MAPK functions as a stress-activated pathway that is stimulated by osmotic stress, DNA damage, and superoxides (Zarubin and Han, 2005). Pathway activation leads to signaling of cell components that regulate proliferation (Wagner and Nebreda, 2009) and apoptosis (Bulavin et al., 1999).

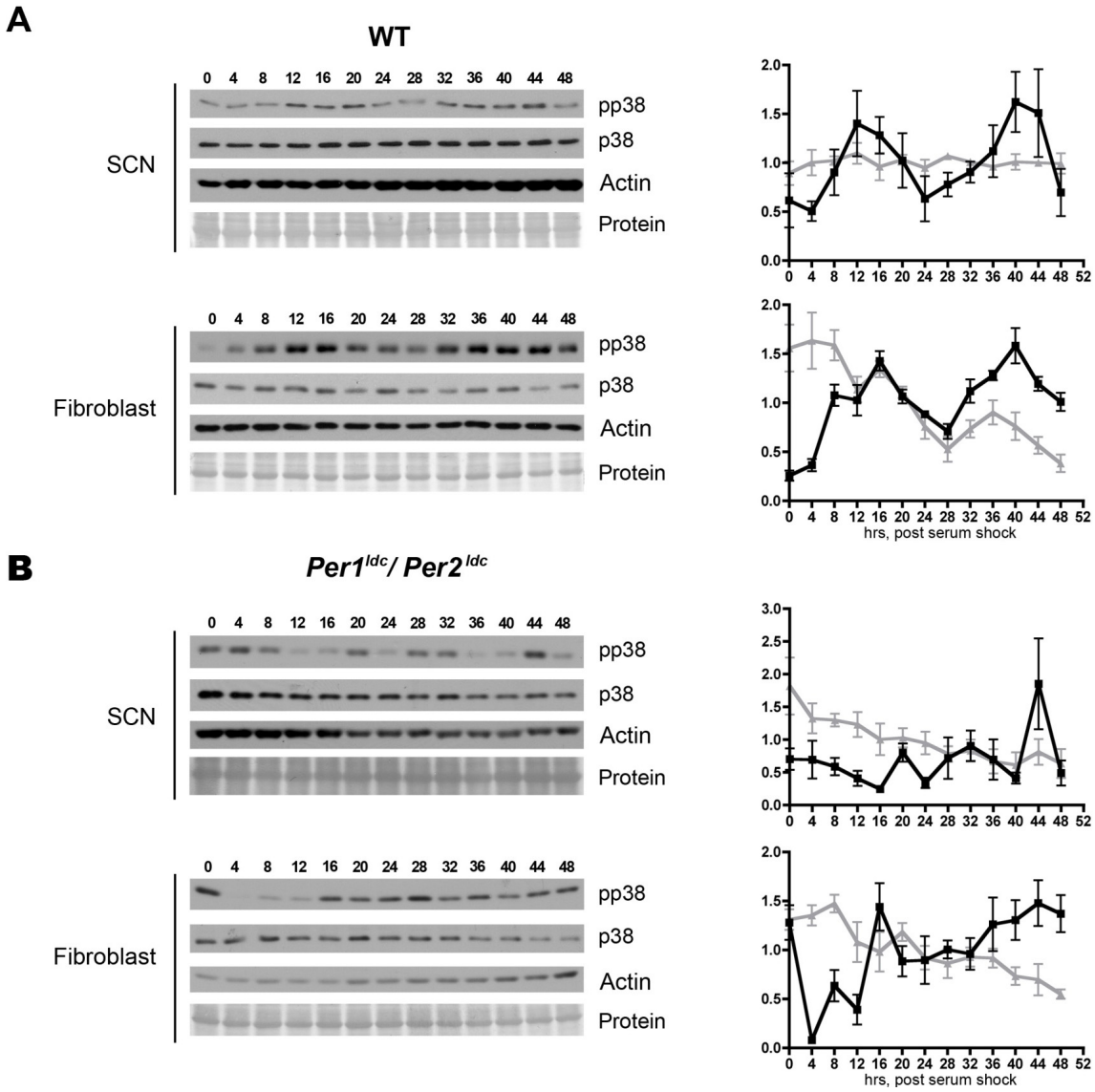
In this chapter, we show that the p38 MAPK is rhythmically activated in cultured mouse fibroblast and SCN cells lines, and that rhythmicity is dependent on a functional circadian oscillator. The overall levels of p38 protein were lower in clock mutant cells,

implying that the circadian oscillator plays an important role in the activation of this pathway.

RESULTS

Previous work in the filamentous fungus Neurospora showed that the *os-2* MAPK, which is related to the p38 MAPK in mammals, is rhythmically activated by the circadian clock and functions within a circadian output pathway to regulate the rhythmic activities of downstream proteins and genes (Lamb et al., 2011; Lamb et al., 2012; Vitalini et al., 2007). We hypothesized that the role of p38 MAPK as an output of the circadian clock is conserved in higher eukaryotes. To assess whether the p38 MAPK is rhythmically phosphorylated in a mammalian system, we used immortalized murine cell lines as an *in vitro* model. Cell lines derived from fibroblasts and the SCN were synchronized by serum shock and periodically harvested over two days. In fibroblasts, the levels of phospho-p38 oscillated with a period of 25.57 (n=3, ± 0.86), with peaks at 16h and 40h after serum shock (Figure 3-1A). In the same fibroblast cultures, the levels of total p38 protein fluctuated arrhythmically over the two days (Figure 3-1A). In SCN cultures, the levels of phospho-p38 oscillated at a period of 25.50 (n=3, ± 0.91), with peaks at 12h and 40h after serum shock (Figure 3-1A). The levels of total p38 protein were also arrhythmic in SCN cells (Figure 3-1A). Alternatively, levels of phospho-p38 in $Per1^{ldc}/Per2^{ldc}$ double mutant cells were erratic and arrhythmic in both SCN and fibroblast cells (Figure 3-1B). These data demonstrated that the p38 MAPK is

Figure 3-1: p38 MAPK activity is rhythmic in synchronous cultures. (A) Both WT mouse fibroblast and SCN cells were synchronized by serum shock and collected at the indicated hours (h) after serum shock. Western blotting was performed on protein extracts and membranes were probed with either anti-phospho-p38, total p38, or actin antibodies. Membranes were stained with amido black to visualize total protein loading and for normalization of signal. Graphs on the right represent the average signal normalized to total protein loaded. The phospho-p38 (black circles) signal was rhythmic in fibroblast and SCN cells as confirmed by statistical best fit to a sine wave with $p<0.0001$; n=3 ± SEM. The total p38 protein (gray triangles) is arrhythmic in fibroblast and SCN cells as confirmed by statistical best fit to a line $p<0.0001$; n=3 ± SEM. (B) *Per1*^{ldc}/*Per2*^{ldc} fibroblasts and SCN cells were synchronized by serum shock and western blotted as described. Graphs on the right represent the average signal of phospho-p38 in *Per1*^{ldc}/*Per2*^{ldc} cells normalized to total protein loaded. The phospho-p38 (black circles) signal was arrhythmic in fibroblast and SCN as confirmed by statistical best fit to a line with $p<0.0001$; n=3 ± SEM. The total p38 protein (gray triangles) is arrhythmic in fibroblast and SCN as confirmed by statistical best fit to a line $p<0.0001$; n=3 ± SEM.


A
WT
0 4 8 12 16 20 24 28 32 36 40 44 48
SCN
pp38
p38
Actin
Protein
Fibroblast
hrs, post serum shock
B
Per1ldc/ Per2ldc

rhythmically phosphorylated in fibroblast and SCN cell cultures, and that the rhythm is dependent on a functional circadian oscillator.

To determine if the *Per1*^{ldc}/ *Per2*^{ldc} mutations had any effect on levels of phosphorylated p38, extracts from wild type and mutant cells were analyzed. Compared to the wild type levels of p38 protein and phosphorylation at the trough in its oscillations at 28h post-serum shock, the levels of phosphorylated p38 were lower in the *Per1*^{ldc}/ *Per2*^{ldc} mutant cultures at several timepoints tested (Figure 3-2). These data suggested that functional *Per1* and *Per2* are required for normal levels of p38 phosphorylation.

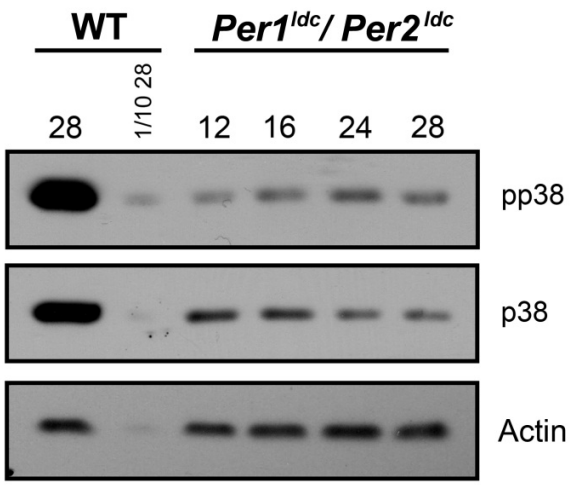


Figure 3-2: p38 MAPK levels are low in the *Per1*^{ldc}*/Per2*^{ldc} double mutant. Protein extracts from both WT and *Per1*^{ldc}*/Per2*^{ldc} fibroblasts were subjected to western blotting and probed with antibodies against phospho-p38, total p38, and actin. A 1/10th fraction of WT protein was loaded as a reference.

DISCUSSION

Previous work (Lamb et al., 2011; Lamb et al., 2012; Vitalini et al., 2007) demonstrating a role for the p38-like MAPK in circadian output led us to investigate if p38 has a similar role in mammals. In this study, we show that p38 MAPK in mouse SCN and fibroblast cell lines has an endogenous rhythm in activation that is dependent on a functional circadian oscillator. The rhythm in p38 activation reaches its apex at 16 h and 40 h post-serum shock in both the fibroblast and SCN cells. In other fibroblast cell lines synchronized by serum shock, this correlates with a phase that roughly precedes the peak in Per1 and Per2 mRNA expression (Akashi and Nishida, 2000; Balsalobre et al., 1998) and lags the peak in Bmal1 mRNA expression (Duffield et al., 2002). There are several reports of diurnal rhythms of p38 activation, that is, daily rhythms that occur as a result of LD cycles, in the chick pineal gland and mouse heart (Chik et al., 2004; Ko et al., 2011). In addition to our findings in Neurospora, endogenous self-sustaining circadian rhythms in p38 activation have been described in the hamster SCN (Pizzio et al., 2003). Our studies provide a validation of p38 rhythms in the hamster SCN, and show that this rhythm can persist in an immortalized cell line derived from mouse SCN tissue. Cell lines derived from the SCN of $Per1^{ldc}/Per2^{ldc}$ mice, in which the circadian oscillator is defective (Bae et al., 2001), confirmed that rhythms in p38 activation require a functional molecular oscillator. These data indicate that p38 is acting as an output pathway of the circadian oscillator. These findings do not, however, rule out the possibility that p38 may be an input pathway to the oscillator, in addition to an output

pathway.

The temporal regulation of p38 activity suggests that targets of the p38 MAPK pathway will also be controlled by the clock, and show time-of-day differences in their respective functions. The p38 MAPK mediates apoptosis in response to several types of cellular stress (Bulavin et al., 1999; Chouinard et al., 2002; Gong et al., 2010; Grethe et al., 2004; Huang et al., 1999; Porras et al., 2004), and our data suggest that cells may be more susceptible to apoptosis at certain phases of the circadian cycle as result of the endogenous rhythm in p38 activity. Attempts have been unsuccessful to uncover a clock-dependent effect on apoptosis (Fu et al., 2002; Gaddameedhi et al., 2012; Gery et al., 2006), by comparing the rate of apoptosis in wild type cells versus cells with defects in clock gene expression, either by mutation or by over-expression of clock genes. In Neurospora, defects in the molecular oscillator do not necessarily compromise signaling through the OS-2 MAPK, the p38 homologue, in response to an acute stress (Vitalini et al., 2007). Interestingly, in Neurospora, the clock "primed" the MAPK pathway for a more rapid and robust response following stress during the peak in circadian MAPK activation (Lamb et al., 2011). These data suggested that the circadian clock "primes" stress response pathways such that a circadian effect on stress response will only be apparent when compared between different phases in the circadian cycle. In experiments that have shown a time-of-day effect on apoptosis in wild type cells (Fu et al., 2002), a dependence on p38 MAPK activity was not tested. Given our data, it is tempting to speculate that the endogenous rhythm in p38 activation in mammalian

peripheral tissue mediates a circadian effect on stress response.

While we hypothesize that p38 lies in an output from the molecular oscillator, several studies implicate p38 MAPK in circadian input. Numerous reports show that inhibition of p38 modulates the properties of the molecular oscillator (Hayashi et al., 2003; Hirota et al., 2008; Yagita et al., 2009), although, in these reports, the drug used for p38 inhibition has off-target effects on kinases known to modulate the circadian clock, notably CKIε (Fabian et al., 2005; Hasegawa and Cahill, 2004). In mammals, the p38 MAPK is a well known mediator of inflammation in response to the cytokine TNFα (Zarubin and Han, 2005). Interestingly, in mouse fibroblasts, TNFα has been shown to induce p-CREB and the expression of Per1, critical biochemical events in clock-resetting (Balsalobre et al., 1998; Best et al., 1999; Tischkau et al., 2003), through a p38-dependent pathway (Petrzilka et al., 2009). Given the potent activation of p38 in response to environmental stimuli and the capability of p38 to modulate the clock, the possibility that p38 is involved in clock-resetting, in addition to circadian output, deserves further consideration.

Our cell culture model also provides a unique tool to understand the molecular mechanisms of circadian p38 signaling. The molecular mechanisms that generate circadian rhythms in the related mammalian ERK MAPK pathway rely on clock-controlled inhibition of upstream components (Antoun et al., 2012; Shimizu et al., 2003), but no mechanism has been suggested for self-sustaining rhythms of p38 MAPK

activation in mammals. Again, in the model organism Neurospora, we have described a mechanism for OS-2 MAPK rhythmicity in which the oscillator components directly regulate transcription of MAPK pathway genes, which in turn, generate rhythms in OS-2 MAPK activation (Lamb et al., 2011). Multiple RNA-seq experiments in mouse liver have shown that Mkk3/Mkk6, the MAPKK's upstream of p38, are rhythmically expressed (Koike et al., 2012; Menet et al., 2012). Furthermore, the expression level of p38 is lower in the $Per1^{ldc}/Per2^{ldc}$ fibroblasts compared to wild type (Fig. 2), supporting the hypothesis that the oscillator influences levels of gene expression in the MAPK pathway. Thus, it is likely that circadian transcription of MAPK pathway components in mammals, similar to Neurospora, plays an important role in generating self-sustaining p38 activity rhythms.

In summary, we have shown that p38 MAPK is rhythmically activated by the circadian clock in mammalian cell culture models for two different tissue types, the master pacemaker and a peripheral tissue. Based on previous work in both Neurospora and mammals, we hypothesize that the circadian clock regulates the gene expression of MAPK pathway components to generate endogenous rhythms in MAPK activation. Lastly, we predict that endogenous rhythms in MAPK activity may prime this pathway to elicit a more robust response to cellular stresses at a specific phase in the circadian cycle.

MATERIALS AND METHODS

Cell culture and conditions

The SCN *mPer2*^{Luc} and *Per1*^{ldc}/ *Per2*^{ldc} cell lines were derived from fetal SCN of *mPer2*^{Luc} (Yoo et al., 2004) and *Per1*^{ldc}/ *Per2*^{ldc} mice (Bae et al., 2001), respectively, and immortalized via the adenovirus E1A gene (Farnell et al., 2011). Cells were maintained on laminin-coated 60mm cell culture dishes (Corning, Corning, NY) in media consisting of MEM (Invitrogen, #10370-021), 10% fetal bovine serum (Hyclone, Thermo Fischer, Waltham, MA), glucose (3000 µg/mL), and L-gluatamine (292 µg/mL). The fibroblast cell lines were derived from wild type (129/SV) or *Per1*^{ldc}/ *Per2*^{ldc} fetal mouse fibroblasts and immortalized by the adenovirus E1A gene. Fibroblast cells were cultured on 60mm cell culture dishes in medium consisting of DMEM high glucose (Invitrogen #11960-044), 10% fetal bovine serum (Hyclone, Thermo Fischer, Waltham, MA) supplemented with L-glutamine (292 µg/mL). Cultures were incubated in 5% CO_2, 37°C and passaged every 3-5 days at a 1:3 ratio. For timecourse samples, SCN cells were synchronized by passaging confluent cultures and harvested over 48 hours at 4 hour intervals. To synchronize fibroblasts, cells were serum shocked according to (Balsalobre et al., 1998) with medium containing 50% horse serum and thereafter maintained on serum free growth medium. Cultures were harvested at 4h intervals for 48 hours.

Immunoblotting

For Western blotting, cells were harvested by trypsinization for 3min with .05% Trypsin/EDTA (Invitrogen #15400). After inactivation with 10% FBS (Hyclone, Thermo Fischer), cells were pelleted by centrifugation at 1000 rpm for 5min. The supernatant was removed and pellets were frozen in liquid nitrogen. To extract protein, 250µl of extraction buffer (20mM Tris pH 7.5; 137mM NaCl; 1% Triton X-100; 10% glycerol; 10mM NaF; 10mM β-glycero-phosphate; 2mM EDTA; 1mM PMSF; 1mM sodium ortho-vanadate; 1x HALT Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL)) was added to cell pellets on ice. While kept cool on an ice bath, the pellets were sonicated using a Branson Sonifier 450 equipped with a microtip for 10s at 10% amplitude. Samples were then placed on ice for 15min before pelleting cell debris at max rpm for 5min at 4°C. An aliquot of protein extract was removed and quantified using the *DC* Protein Assay (Bio-Rad; Hercules, CA). 30µg of protein was boiled for 5min with 1x Laemmli buffer before being separated via 10% SDS-PAGE. Protein was transferred from gels to Immobolin-P PVDF membrane (EMD Millipore, Billerica, MA) and immunoblotted according to antibody protocols. For detection of phospho-p38, membranes were probed with mouse anti-phospho-p38 primary (#9216 Cell Signaling, Beverly, MA), and anti-mouse-HRP secondary (#170-6516 BioRad, Hercules, CA) antibodies. For detection of total p38, membranes were probed with rabbit anti-p38 primary (#9216 Cell Signaling, Beverly, MA), and anti-rabbit-HRP secondary (#170-6515 BioRad, Hercules, CA) antibodies. For detection of actin, membranes were probed

with mouse anti-actin primary (#A4700 Sigma-Aldrich, St Louis, MO) Blots were visualized on X-ray film (Phenix, Candler, NC) with Super Signal West Fempto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL)

Statistical analysis

Statistical analysis of rhythmic data was performed as in (Lamb et al., 2011). Briefly, using Prism software (GraphPad Software, San Diego, CA), comparison of rhythmic data to either a line or sine wave with nonlinear regression and Akaike's information criteria established if a rhythm was significantly more similar to a sine wave than a line. The error bars in graphs represent standard error mean of at least three biological replicates.

CHAPTER IV

CONCLUSION

ENDOGENOUS RHYTHMS OF MAPK ACTIVATION PRIME THE ORGANISM FOR A STRESS RESPONSE

Priming of the osmotic stress response in Neurospora

In Chapter II, I presented the first model to explain how the p38 MAPK pathway acts as an output pathway to temporally regulate stress response. I showed that the endogenous rhythm of p-OS-2 MAPK, which peaks during the day and is less active at night, primes the organism for a more robust stress response during the day, a time that is presumably most advantageous to the organism. When the OS MAPK is activated by an acute osmotic stress, it engages an adaptation response through the production of glycerol, which protects the organism from the deleterious effects of severe osmotic gradients. In our experiments, an osmotic stress administered during the daytime resulted in more rapid and intense production of glycerol when compared to the night time response from an identical stress. The daytime correlates with the peak in the endogenous p-OS-2 rhythm and occurs at a time that the organism would presumably encounter similar types of stress from the sun. No difference in glycerol production between day and night was

observed in a strain with arrhythmic p-OS-2. These data demonstrated that rhythms in p-OS-2 were required for the priming of stress response. In the absence of an osmotic stress, no observable circadian rhythm in glycerol production was detected, indicating that the rhythms in OS-2 activation were not merely driving production of glycerol, but preparing the organism for a stress when it is most likely to occur. This priming effect is consistent with the idea that the circadian clock provides an advantage to the organism: it would be inefficient and wasteful to drive the production of glycerol in anticipation of daytime when no immediate stress exists. However, it is interesting that no glycerol is produced even though OS-2 is activated by the clock. One explanation of this observation is the fact that endogenous levels of p-OS-2 are much lower than levels induced by an acute stress (Vitalini et al., 2007). Perhaps the endogenous levels never accumulate to a critical threshold for the production of glycerol. Interestingly this phenomenon is seen in other rhythmic MAPK's. For example, the levels of p-ERK resulting from photic stimulus are much higher than the p-ERK levels observed in the endogenous rhythm (Obrietan et al., 1998). Another explanation for the priming of glycerol production, as opposed to driven rhythms, is the gating of stress response. While the OS MAPK may be poised to respond to stress through the clock influence, it is possible that a mechanism may keep glycerol production repressed until it is challenged with a stress. Regardless, it is assumed that somehow the clock activation of OS-2, in this regard, gives the organism a "head start" in responding to the stress.

Does p38 mediate a priming of stress response in mammals

Since the mammalian p38 MAPK pathway is a rhythmic stress response pathway in our cell lines, one would expect a similar circadian priming effect on p38-mediated stress responses. In peripheral tissues it could be hypothesized that rhythms in p38 would contribute somehow to the function of that tissue, or anticipate daily stress that tissue might encounter. The only reported example of circadian regulation of a stress response is related to UV-induced DNA damage (Kang et al., 2011). This report demonstrated that the nucleotide excision repair gene XPA is rhythmically expressed by the circadian clock, and as a result, fibroblasts more efficiently repair UV-induced DNA damage during certain phases of the circadian cycle. p38 MAPK responds to stresses such as osmotic shock, heat shock, inflammatory cytokines, DNA damage and ROS (Zarubin and Han, 2005). In response to those signals, p38 generally has a role in cell survival through the regulation of apoptosis (Porras et al., 2004; Zarubin and Han, 2005). Since our mouse fibroblast cell line provided a model of a peripheral tissue in which p38 activity is known to be rhythmic, I was interested in testing if p38 activation rhythms prime these cells for a more robust stress response at the peak in p38 activation. In my experimental design, UV-B-induced DNA damage was used as the stress perturbation. UV radiation is known to activate all three MAPK families, however, the UV-B bandwidth more specifically activates p38 MAPK (Chouinard et al., 2002). After UV induction, the p38 MAPK is known to phosphorylate key regulatory sites on p53, a master regulator of cell survival, which in turn can induce apoptosis (Bulavin et al.,

1999; Jinlian et al., 2007; Porras et al., 2004). Knowing that the p38-mediated stress response to UV-induced DNA damage is apoptosis, I designed experiments to assay the fibroblasts' susceptibility to apoptosis after UV treatment at the circadian times correlating to the peak and trough in p38 activation rhythms. Unfortunately, due to technical issues, I have not yet found an approach that can reliably test this hypothesis.

The same group that identified a rhythm in XPA expression has undertaken similar approaches to identify a circadian effect on fibroblast survival after UV-induced DNA damage (Gaddameedhi et al., 2012). In their experimental design, they compared cell survival after UV stress in wild type fibroblasts to cells with null clock mutations (Gaddameedhi et al., 2012). They saw no difference between the two genotypes, and their conclusion was that the clock has no effect on UV-induced apoptosis (Gaddameedhi et al., 2012). Our efforts in Neurospora to define the priming of stress responses by the circadian clock have highlighted a flaw in the experimental design of Gaddameedhi et al. (2012). The circadian oscillators in the wild type fibroblasts were not synchronized for the experiment in Gaddameedhi et al. (2012), and, therefore, the endogenous oscillators in individual cells were either not cycling or out of synchrony with each other, preventing a coherent ensemble rhythm in the culture. In Neurospora, the levels of glycerol after an osmotic shock in the clock deficient Δ*frq* strain were intermediate between the day and night levels of glycerol observed in the wild type strain (Lamb et al., 2011). Similar levels of glycerol might be predicted in an arrhythmic wild type culture grown in constant light (LL). Without an endogenous circadian rhythm

in the wild type cells, the priming effect on the glycerol stress response would be negligible compared to clock deficient cultures. This prediction casts doubt on the conclusion of Gaddameedhi et al. (2012) that the clock has no effect on cell survival after UV treatment in mammalian fibroblasts. With these data in mind, it is important to compare the stress response from two opposing circadian times when attempting to define a circadian effect on stress response.

Furthermore, to look for a circadian effect on stress response, the types of cells that are utilized should be carefully considered. While fibroblasts provide a generalized model of peripheral tissues, differentiated cells like keratinocytes are more likely to encounter UV stress in the whole animal and may provide a better model to test cell survival after UV stress (Chouinard et al., 2002). However, it is not known if p38 activity is regulated by the circadian clock in keratinocytes. There are several cell types in which p38 has a vital role in the biological function of the cell, *e.*g. immune cells (Ashwell, 2006). In immune cells, p38 mediates the production of cytokines and immune receptors in response to antigenic signals (Ashwell, 2006). It is known that self-sustaining circadian oscillators exist in some immune cells (Boivin et al., 2003; Keller et al., 2009), and, therefore, it is likely that a circadian rhythm in p38 MAPK activity would have a profound effect in these cells.

CLOCK REGULATION OF THE PHOSPHO-RELAY IN NEUROSPORA

As demonstrated in Chapter II, a circadian rhythm in *hpt-1* mRNA and protein accumulation peaks during the subjective night and requires a functional FWO. Based on the related HOG pathway in yeast, the gene *hpt-1* in Neurospora relays a signal from the sensory histidine kinases to response regulator proteins, which are responsible for signaling to components downstream of the phospho-relay (Posas et al., 2000). While the yeast genome encodes only one histidine kinase, the Neurospora genome, like many other filamentous fungi, encodes 11 histidine kinases (Borkovich et al., 2004). The histidine kinases in Neurospora contain diverse protein domains and putatively sense various environmental stimuli (Borkovich et al., 2004). Because the histidine kinases in Neurospora are predicted to signal through a single histidine phosphotransferase, the gene *hpt-1* represents a significant bottle neck in the phospho-relay pathway (Catlett et al., 2003). Clock regulation of *hpt-1* implies that the circadian clock may modulate the sensitivity of the phospho-relay to environmental stimuli at different times of the day.

In order to better understand the clock regulation of the phospho-relay, I have investigated the molecular basis for the circadian rhythm of *hpt-1* expression. Identification of the genomic targets of the WCC revealed that many transcription factor genes are regulated by the WCC (Smith et al., 2010). These first-tier transcription factors are transcriptionally activated by the WCC in response to light, and many of these transcription factors are rhythmically expressed in constant conditions (Smith et

al., 2010). In unpublished data from our lab, ChIP-seq was used to identify the genomic targets of the first-tier transcription factors regulated by the WCC. A query of this dataset showed that several first-tier transcription factors bound to the promoter of the *hpt-1* gene (Figure A-1). Analysis of *hpt-1* mRNA accumulation in strains harboring a KO mutation of these transcription factors showed that rhythms in *hpt-1* mRNA accumulation persisted in the mutant strains (Figure A-2). While *hpt-1* mRNA rhythmically accumulates in the mutant strains, it is possible that these rhythms in *hpt-1* expression may have defects in period or phase that are impossible to detect using timepoints at intervals of 4 hrs. An *hpt-1:luc* transcriptional reporter strain is currently in development to provide real-time monitoring of *hpt-1* transcription. Using this strain, minor variations in the period or phase of *hpt-1* transcription should be detectable. Furthermore, it is possible that multiple transcription factors in tandem generate rhythms in *hpt-1* expression. In order to test this, *hpt-1* expression rhythms will be assayed using the *hpt-1:luc* reporter construct in strains containing double knockout mutation of candidate transcription factors.

Interestingly, histidine kinase genes were also targets of the WCC after a light pulse (Smith et al., 2010). The genes *ncu07221* and *ncu00939*, both PER-ARNT-SIM (PAS) domain-containing histidine kinases, were bound by the WCC in response to a light pulse, and the accumulation of their mRNA was induced by light. Our lab has validated the binding of the WCC to these candidate gene promoters after a light pulse (Figure A-3). Furthermore, the mRNA accumulation of *ncu07221* was rhythmic in constant

conditions (Figure A-4), but expression of *ncu00939* was arrhythmic. Because these histidine kinase genes were targeted by the WCC and were rhythmically expressed, these data suggest that histidine kinase proteins may participate in clock regulation of phospho-relay signaling.

TRANSCRIPTIONAL REGULATION AS MECHANISM FOR RHYTHMIC MAPK ACTIVATION

Transcriptional regulation of MAPK components in Neurospora

In Chapter II, I described our efforts in Neurospora to define a molecular mechanism for the rhythmicity of p-OS-2 MAPK. This model predicts that the transcriptional regulation by the circadian clock of OS pathway components, the OS-4 MAPKKK and phospho-relay component HPT-1, modulates the abundance of these gene products which in turn lead to rhythms in p-OS-2. Also, this model posits that the transcriptional regulation by the circadian clock segregates the expression of positive and negative regulators into opposite phases, thereby creating resonance of their activities rather than dissonance. This resonance tunes the levels of basal signal transduction in the OS pathway and produces endogenous rhythms of p-OS-2. While the clock impinges on the OS pathway at two positions, we cannot yet fully test the necessity and sufficiency of these two clock inputs on the rhythmicity of p-OS-2. Unfortunately, we do not yet have genetic tools to eliminate the clock regulation of *hpt-1* expression. However, we did

show that rhythmic expression of *os-4* is required for p-OS-2 rhythms, but were unable to test for sufficiency due to the lack of genetic tools to disrupt *hpt-1*. Currently, the Bell-Pedersen lab is developing a strain in which the expression of the *hpt-1* is arrhythmic. Using this tool, we can determine if *os-4* expression rhythms are sufficient to generate rhythms in OS-2 activity, and, likewise, if *hpt-1* expression rhythms are necessary for p-OS-2 rhythms.

Transcriptional regulation of MAPK components in mammalian peripheral tissue

As a validation of our model for the mechanism that controls p-OS-2 rhythms, it would be interesting to test mammalian cell lines for rhythmic expression of genes in the p38 MAPK cascade. I have attempted to look for rhythmic mRNA expression of all MAPK cascade components upstream of p38 in rhythmic fibroblast cells. Unfortunately, preliminary validation experiments showed that clock genes were not oscillating in the samples, and resources were not available to repeat the experiments. Fortunately, numerous datasets from RNA-seq experiments are available in a variety of mammalian tissues. Review of RNA-seq datasets shows that mRNA accumulation of Mkk3 and Mkk6, the two MAPKK's upstream of p38, is rhythmic in the mouse liver (Koike et al., 2012; Menet et al., 2012). Also, numerous components upstream and downstream of the p38 MAPK showed rhythmic mRNA accumulation in mouse macrophages (Keller et al., 2009). These data provide promising indications that p38 is rhythmically activated in other mammalian tissues, and that these rhythms are the result of a transcriptional

mechanism similar to what we have modeled in Neurospora.

THE ROLE OF SIGNALING COMPONENTS DOWNSTREAM OF THE P38 MAPK CIRCADIAN OUTPUT PATHWAY

As a signaling molecule, the MAPK relies on interactions with a network of downstream components to elicit a biological effect. Components downstream of the MAPK provide the opportunity to integrate signals from other MAPK signaling pathways or, conversely, to confer signaling specificity. For example, the MAPK-interacting kinase MSK-1 integrated signals from both the ERK and p38 MAPK after photic stimulation in the mouse SCN (Butcher et al., 2005). Alternatively, a related MAPK-interacting kinase, RSK-1, specifically propagated an ERK-mediated signal in response to neurotrophins in the mouse SCN (Arthur et al., 2004). In this regard, investigation of downstream effector molecules will be important to understand the specificity of circadian signal transduction. In Neurospora, the Bell-Pedersen lab has made significant progress toward understanding interaction of OS-2 MAPK with downstream components in regards to circadian signaling. We have shown that the transcription factor ASL-1, similar to the mammalian ATF-1, regulates the rhythmic expression of several ccg's known to be downstream of OS-2 MAPK (Lamb et al., 2012). It remains unknown if the OS-2 MAPK rhythmically phosphorylates this transcription factor to confer rhythmicity to ccg's. Also, in studies that are currently unpublished, the Bell-Pedersen lab has shown that a rhythmic signal from OS-2 is propagated through the OS-2/RCK-2/eEF-2

signaling cassette, a pathway known to regulate protein translation. Future studies in both Neurospora and mammalian cell lines will help clarify the role of downstream regulatory components.

THE FUNCTION OF P38 IN CIRCADIAN INPUT PATHWAYS

The use of p38 pharmacological inhibitors

p38 MAPK has been implicated as an input pathway to circadian oscillators in numerous reports that were reviewed in Chapter I. The most consistent evidence that p38 modulates the molecular oscillator is data showing that the period of circadian rhythms, either an output rhythm like melatonin production (Hasegawa and Cahill, 2004; Hayashi et al., 2003) or oscillations of clock genes (Hirota et al., 2008; Yagita et al., 2009), were lengthened after treatment with a pharmacological p38 inhibitor. The inhibitors used in these reports, SB 203580 and SB 212190, are known to have off-target effects on a multitude of kinases (Fabian et al., 2005). Indeed, Hasegawa and colleagues even described the non-specific inhibitory effects of SB 203580 on JNK and CKI (Hasegawa and Cahill, 2004), which are both known to modulate the period of the circadian oscillator in animals (Isojima et al., 2009; Yoshitane et al., 2012). Furthermore, an inhibitor of JNK was found to phenocopy the effects of the p38 inhibitor (Hasegawa and Cahill, 2004). These data cast serious doubt on any conclusions directed toward the specific function of p38 MAPK that were derived from the usage of this inhibitor.

Nevertheless, investigators continue to use this drug, and the conclusions have yet to be confirmed by other methods.

The mammalian cell lines used in Chapter III provide an excellent tool to test the effect of p38 activity on clock gene oscillations. The SCN cell line used in Chapter III is derived from a mouse with a *mPer2:luc* reporter, which is a useful tool to monitor the real-time oscillations of the circadian clock. A fibroblast cell line is available from this same mouse strain. Experiments are currently underway to test if a more potent and specific inhibitor of p38, VX-745 (Bagley et al., 2007; Fabian et al., 2005), will cause a similar lengthening of the period in clock gene expression. Preliminary data from these experiments suggests that the period of clock gene rhythms is lengthened. While the usage of a specific inhibitor is a straight-forward preliminary experiment, follow-up experiments should use siRNA to knock down the expression of p38 for a more rigorous approach.

Can p38 MAPK pathway reset the circadian oscillator

In the most convincing report describing p38 as a circadian input pathway, treatment of mouse fibroblasts with the inflammatory cytokine TNFα, a potent activator of p38, resulted in the phosphorylation of CREB and upregulation of Per1, two hallmark biochemical events in clock-resetting (Balsalobre et al., 1998; Petrzilka et al., 2009). Induction of p-CREB and Per1 by TNFα was blocked by a p38 pharmacological

inhibitor (Petrzilka et al., 2009). Based on these data, it is hypothesized that other p38-activating stimuli can reset the circadian oscillator. Interestingly, damaging doses of UV light, which potently activate p38 MAPK, have been reported to produce phase advances of clock gene rhythms in mouse fibroblasts; however, the contribution of p38 to this effect was not tested (Gamsby et al., 2009). The mouse fibroblast cell lines used in my studies can be a vital tool to test if p38-inducing stimuli can reset the clock. If p38-activating stimuli, such as osmotic shock, UV light, and ROS, do induce immediate early gene expression, the dependency on p38 activity could be examined through siRNA knock down or by pharmacological inhibition. Similarly, it is uncertain if known resetting stimuli require the activity of p38 to regulate clock gene expression. The most straight-forward experiment would test if *bona fide* resetting stimuli (serum shock, forskolin, growth factors, etc. (Balsalobre et al., 1998)) can induce p-CREB and immediate early gene expression in the presence of a p38 inhibitor. These experiments would test the hypothesis that the p38 MAPK resides in a circadian input pathway to modulate the activity of the circadian oscillator in peripheral tissue.

In one of the most interesting descriptions of p38 as an input pathway to the clock, rhythms in p-p38 were observed in a mouse heart that possessed a tissue-specific defect in the circadian oscillator (Ko et al., 2011). In this mutant mouse, the circadian clock was functional throughout the organism except in the heart, where null alleles of clock genes were specifically expressed (Ko et al., 2011). Their conclusion was that p38 continued to receive a humeral signal from the SCN, even though the oscillator in the

heart was defective; therefore, p38 lies in a circadian input pathway (Ko et al., 2011). Unfortunately, the mice in this experiment were housed in LD conditions instead of DD conditions, and it seems likely that the rhythm in p-p38 could be driven by light or a behavioral rhythm (Ko et al., 2011). To test if the p38 activity rhythm in a clock-defective heart is driven by light input, this experiment should be repeated in DD conditions, although this would not eliminate the possibility that the rhythm is driven by behavior. The cell culture system used in my experiments could be used to confirm this finding. The SCN cell line can be co-cultured with fibroblasts, and a diffusible signal from the SCN cells entrains the oscillators in the fibroblasts (Allen et al., 2001). If fibroblasts derived from a *Per1*^{ldc}/*Per2*^{ldc} mouse, in which the circadian oscillator is defective, were co-cultured with SCN cells, the presence of p-p38 rhythms could be examined. This would provide a system that lacks the influence of behavior to test the ability of p38 to relay a diffusible signal from the SCN. Although such a cell line does not currently exist, a cardiomyocyte cell line derived from the heart of *Per1*^{ldc}/*Per2*^{ldc} mice could also be used to confirm a tissue specific function of p38 as an input pathway in an SCN co-culture.

Is p38 an input to the Neurospora clock

In Vitalini et al. (2007), experiments were performed to verify that mutations in the OS pathway did not have an effect on the endogenous oscillations of FRQ . Those experiments provided a confirmation that OS-2 functions in a circadian output pathway

and not an input pathway. In that experimental setup, FRQ levels were assayed in cultures collected at 4h intervals over a circadian time course. This experiment will not provide sufficient temporal resolution to detect a small period defects in FRQ rhythms. At the time this experiment was performed, luciferase reporters were not readily available in Neurospora. However, at present, the use of a FRQ:LUC translational fusion reporter is commonplace to assay the rhythmicity of the circadian oscillator in real-time (Gooch et al., 2008). The most straight-forward experiment would examine FRQ:LUC rhythms in mutants of the OS pathway. Additionally, p38 inhibitors like VX-745 can selectively inhibit the activity of the mammalian p38 MAPK, and, due to the similarity between p38 and OS-2, it is likely that this inhibitor will work in Neurospora to inhibit the kinase activity of OS-2. If VX-745 can inhibit OS-2, this drug could be applied to the growth medium of a wild type strain to test for an effect on FRQ:LUC rhythms. Additionally, the same FRQ:LUC strain should be used to test whether OS-2-activating stimuli, such as an osmotic shock, has an effect on the rhythmicity of FRQ protein, an indication that OS-2 can phosphorylate clock proteins. These experiments will sufficiently test the hypothesis that OS-2 MAPK is a circadian input pathway, in addition to its role as a circadian output pathway.

REFERENCES

Akashi, M., and Nishida, E. (2000). Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes & Development *14*, 645-649.

Akashi, M., Hayasaka, N., Yamazaki, S., and Node, K. (2008). Mitogen-activated protein kinase is a functional component of the autonomous circadian system in the suprachiasmatic nucleus. The Journal of Neuroscience *28*, 4619-4623.

Allen, G., Rappe, J., Earnest, D.J., and Cassone, V.M. (2001). Oscillating on borrowed time: Diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. The Journal of Neuroscience *21*, 7937-7943.

Angell, R.M., Bamborough, P., Cleasby, A., Cockerill, S.G., Jones, K.L., Mooney, C.J., Somers, D.O., and Walker, A.L. (2008). Biphenyl amide p38 kinase inhibitors 1: Discovery and binding mode. Bioorganic & Medicinal Chemistry Letters *18*, 318-323.

Anjum, R., and Blenis, J. (2008). The RSK family of kinases: Emerging roles in cellular signalling. Nature Reviews Molecular Cell Biology *9*, 747-758.

Antoun, G., Cannon, P.B., and Cheng, H.-Y.M. (2012). Regulation of MAPK/ERK signaling and photic entrainment of the suprachiasmatic nucleus circadian clock by Raf kinase inhibitor protein. The Journal of Neuroscience *32*, 4867-4877.

Aronson, B.D., Johnson, K.A., Loros, J.J., and Dunlap, J.C. (1994). Negative feedback defining a circadian clock: Autoregulation of the clock gene frequency. Science *263*, 1578-1584.

Arthur, J.S., Fong, A.L., Dwyer, J.M., Davare, M., Reese, E., Obrietan, K., and Impey, S. (2004). Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. The Journal of Neuroscience *24*, 4324-4332.

Ashwell, J.D. (2006). The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nature Reviews Immunology *6*, 532-540.

Athos, J., Impey, S., Pineda, V.V., Chen, X., and Storm, D.R. (2002). Hippocampal CRE-mediated gene expression is required for contextual memory formation. Nature Neuroscience *5*, 1119-1120.

Atkins, C.M., Selcher, J.C., Petraitis, J.J., Trzaskos, J.M., and Sweatt, J.D. (1998). The MAPK cascade is required for mammalian associative learning. Nature Neuroscience *1*, 602-609.

Bae, K., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., and Weaver, D.R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron *30*, 525-536.

Bagley, M.C., Davis, T., Dix, M.C., Rokicki, M.J., and Kipling, D. (2007). Rapid synthesis of VX-745: p38 MAP kinase inhibition in Werner syndrome cells. Bioorganic & Medicinal Chemistry Letters *17*, 5107-5110.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: An update. Biochemical Journal *371*, 199-204.

Baker, C.L., Kettenbach, A.N., Loros, J.J., Gerber, S.A., and Dunlap, J.C. (2009). Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the Neurospora circadian clock. Molecular Cell *34*, 354-363.

Balsalobre, A., Damiola, F., and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell *93*, 929-937.

Balsalobre, A., Marcacci, L., and Schibler, U. (2000). Multiple signaling pathways elicit circadian gene expression in cultured rat-1 fibroblasts. Current Biology *10*, 1291-1294.

Banno, S., Noguchi, R., Yamashita, K., Fukumori, F., Kimura, M., Yamaguchi, I., and Fujimura, M. (2007). Roles of putative His-to-Asp signaling modules HPT-1 and RRG-2, on viability and sensitivity to osmotic and oxidative stresses in Neurospora crassa. Current Genetics *51*, 197-208.

Bardwell, L. (2006). Mechanisms of MAPK signalling specificity. Biochemical Society Transactions *34*, 837.

Barr, R.K., and Bogoyevitch, M.A. (2001). The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). The International Journal of Biochemistry & Cell Biology *33*, 1047-1063.

Bell-Pedersen, D., Dunlap, J.C., and Loros, J.J. (1992). The Neurospora circadian clock-controlled gene, ccg-2, is allelic to eas and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. Genes & Development *6*, 2382-2394.

Bell-Pedersen, D., Shinohara, M.L., Loros, J.J., and Dunlap, J.C. (1996). Circadian clock-controlled genes isolated from Neurospora crassa are late night- to early morning-specific. Proceedings of the National Academy of Sciences of the United States of America *93*, 13096-13101.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., and Zoran, M.J. (2005). Circadian rhythms from multiple oscillators: Lessons from diverse organisms. Nature Reviews Genetics *6*, 544-556.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y.*, et al.* (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proceedings of the National Academy of Sciences of the United States of America *98*, 13681-13686.

Bennett, L.D., Beremand, P., Thomas, T.L., and Bell-Pedersen, D. (2013). Circadian activation of the mitogen-activated protein kinase MAK-1 facilitates rhythms in clock-controlled genes in Neurospora crassa. Eukaryotic Cell *12*, 59-69.

Bentley, D.R., Balasubramanian, S., Swerdlow, H.P., Smith, G.P., Milton, J., Brown, C.G., Hall, K.P., Evers, D.J., Barnes, C.L., Bignell, H.R.*, et al.* (2008). Accurate whole human genome sequencing using reversible terminator chemistry. Nature *456*, 53-59.

Berman-Frank, I., Lundgren, P., and Falkowski, P. (2003). Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Research in Microbiology *154*, 157-164.

Best, J.D., Maywood, E.S., Smith, K.L., and Hastings, M.H. (1999). Rapid resetting of the mammalian circadian clock. The Journal of Neuroscience *19*, 828-835.

Blum, S., Moore, A.N., Adams, F., and Dash, P.K. (1999). A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. The Journal of Neuroscience *19*, 3535-3544.

Bogoyevitch, M.A. (2006). The isoform-specific functions of the c-Jun N-terminal Kinases (JNKs): Differences revealed by gene targeting. Bioessays *28*, 923-934.

Boivin, D.B., James, F.O., Wu, A., Cho-Park, P.F., Xiong, H., and Sun, Z.S. (2003). Circadian clock genes oscillate in human peripheral blood mononuclear cells. Blood *102*, 4143-4145.

Borkovich, K.A., Alex, L.A., Yarden, O., Freitag, M., Turner, G.E., Read, N.D., Seiler, S., Bell-Pedersen, D., Paietta, J., Plesofsky, N.*, et al.* (2004). Lessons from the genome sequence of Neurospora crassa: Tracing the path from genomic blueprint to multicellular organism. Microbiology and Molecular Biology Reviews *68*, 1-108.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A.J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell *79*, 59-68.

Bozek, K., Rosahl, A.L., Gaub, S., Lorenzen, S., and Herzel, H. (2010). Circadian transcription in liver. Biosystems *102*, 61-69.

Bray, M.S., Shaw, C.A., Moore, M.W.S., Garcia, R.A.P., Zanquetta, M.M., Durgan, D.J., Jeong, W.J., Tsai, J.-Y., Bugger, H., Zhang, D.*, et al.* (2008). Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. American Journal of Physiology - Heart and Circulatory Physiology *294*, H1036-H1047.

Brunner, M., and Schafmeier, T. (2006). Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and Neurospora. Genes & Development *20*, 1061-1074.

Brunner, M., and Káldi, K. (2008). Interlocked feedback loops of the circadian clock of Neurospora crassa. Molecular Microbiology *68*, 255-262.

Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace, A.J. (1999). Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. The EMBO journal *18*, 6845-6854.

Butcher, G.Q., Doner, J., Dziema, H., Collamore, M., Burgoon, P.W., and Obrietan, K. (2002). The p42/44 mitogen-activated protein kinase pathway couples photic input to circadian clock entrainment. The Journal of Biological Chemistry *277*, 29519-29525.

Butcher, G.Q., Lee, B., and Obrietan, K. (2003). Temporal regulation of light-induced extracellular signal-regulated kinase activation in the suprachiasmatic nucleus. Journal of Neurophysiology *90*, 3854-3863.

Butcher, G.Q., Lee, B., Hsieh, F., and Obrietan, K. (2004). Light- and clock-dependent regulation of ribosomal S6 kinase activity in the suprachiasmatic nucleus. European Journal of Neuroscience *19*, 907-915.

Butcher, G.Q., Lee, B., Cheng, H.Y., and Obrietan, K. (2005). Light stimulates MSK1 activation in the suprachiasmatic nucleus via a PACAP-ERK/MAP kinase-dependent mechanism. The Journal of Neuroscience *25*, 5305-5313.

Cahill, G.M. (2002). Clock mechanisms in zebrafish. Cell and Tissue Research *309*, 27-34.

Cao, R., Lee, B., Cho, H.Y., Saklayen, S., and Obrietan, K. (2008). Photic regulation of the mTOR signaling pathway in the suprachiasmatic circadian clock. Molecular and Cellular Neuroscience *38*, 312-324.

Cao, R., Li, A., Cho, H.Y., Lee, B., and Obrietan, K. (2010). Mammalian target of rapamycin signaling modulates photic entrainment of the suprachiasmatic circadian clock. The Journal of Neuroscience *30*, 6302-6314.

Cao, R., Anderson, F.E., Jung, Y.J., Dziema, H., and Obrietan, K. (2011). Circadian regulation of mammalian target of rapamycin signaling in the mouse suprachiasmatic nucleus. Neuroscience.

Cao, R., Butcher, G.Q., Karelina, K., Arthur, J.S., and Obrietan, K. (2013). Mitogen- and stress-activated protein kinase 1 modulates photic entrainment of the suprachiasmatic circadian clock. European Journal of Neuroscience *37*, 130-140.

Catlett, N.L., Yoder, O.C., and Turgeon, B.G. (2003). Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryotic Cell *2*, 1151-1161.

Cermakian, N., Pando, M.P., Thompson, C.L., Pinchak, A.B., Selby, C.P., Gutierrez, L., Wells, D.E., Cahill, G.M., Sancar, A., and Sassone-Corsi, P. (2002). Light induction of a vertebrate clock gene involves signaling through blue-light receptors and MAP kinases. Current Biology *12*, 844-848.

Cha, J., Huang, G., Guo, J., and Liu, Y. (2007). Posttranslational control of the Neurospora circadian clock. Paper presented at: Cold Spring Harbor Symposia on Quantitative Biology (Cold Spring Harbor Laboratory Press).

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37 - 40.

Chansard, M., Molyneux, P., Nomura, K., Harrington, M.E., and Fukuhara, C. (2007). c-Jun N-terminal kinase inhibitor SP600125 modulates the period of mammalian circadian rhythms. Neuroscience *145*, 812-823.

Chen, C.-H., DeMay, B.S., Gladfelter, A.S., Dunlap, J.C., and Loros, J.J. (2010). Physical interaction between VIVID and white collar complex regulates photoadaptation in Neurospora. Proceedings of the National Academy of Sciences of the United States of America *107*, 16715-16720.

Chen, L.W., Chen, P.H., Chang, W.J., Wang, J.S., Karin, M., and Hsu, C.M. (2007). IKappaB-kinase/nuclear factor-kappaB signaling prevents thermal injury-induced gut damage by inhibiting c-Jun NH2-terminal kinase activation. Critical Care Medicine *35*, 1332-1340.

Cheng, H.-Y.M., Obrietan, K., Cain, S.W., Lee, B.Y., Agostino, P.V., Joza, N.A., Harrington, M.E., Ralph, M.R., and Penninger, J.M. (2004). Dexras1 potentiates photic and suppresses nonphotic responses of the circadian clock. Neuron *43*, 715-728.

Cheng, H.-Y.M., Dziema, H., Papp, J., Mathur, D.P., Koletar, M., Ralph, M.R., Penninger, J.M., and Obrietan, K. (2006). The molecular gatekeeper Dexras1 sculpts the photic responsiveness of the mammalian circadian clock. The Journal of Neuroscience *26*, 12984-12995.

Cheng, H.-Y.M., Papp, J.W., Varlamova, O., Dziema, H., Russell, B., Curfman, J.P., Nakazawa, T., Shimizu, K., Okamura, H., Impey, S.*, et al.* (2007). microRNA modulation of circadian-clock period and entrainment. Neuron *54*, 813-829.

Cheng, P., Yang, Y., Heintzen, C., and Liu, Y. (2001a). Coiled-coil domain-mediated FRQ–FRQ interaction is essential for its circadian clock function in Neurospora. The EMBO journal *20*, 101-108.

Cheng, P., Yang, Y., and Liu, Y. (2001b). Interlocked feedback loops contribute to the robustness of the Neurospora circadian clock. Proceedings of the National Academy of Sciences of the United States of America *98*, 7408-7413.

Cheng, P., Yang, Y., Wang, L., He, Q., and Liu, Y. (2003). WHITE COLLAR-1, a multifunctional Neurospora protein involved in the circadian feedback loops, light sensing, and transcription repression of wc-2. Journal of Biological Chemistry *278*, 3801-3808.

Chik, C.L., Mackova, M., Price, D., and Ho, A.K. (2004). Adrenergic regulation and diurnal rhythm of p38 mitogen-activated protein kinase phosphorylation in the rat pineal gland. Endocrinology *145*, 5194-5201.

Chouinard, N., Valerie, K., Rouabhia, M., and Huot, J. (2002). UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. Biochemical Journal *365*, 133-145.

Coogan, A.N., and Piggins, H.D. (2003). Circadian and photic regulation of phosphorylation of ERK1/2 and Elk-1 in the suprachiasmatic nuclei of the syrian hamster. The Journal of Neuroscience *23*, 3085-3093.

Corbit, K.C., Trakul, N., Eves, E.M., Diaz, B., Marshall, M., and Rosner, M.R. (2003). Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. Journal of Biological Chemistry *278*, 13061-13068.

Correa, A., Lewis, Z.A., Greene, A.V., March, I.J., Gomer, R.H., and Bell-Pedersen, D. (2003). Multiple oscillators regulate circadian gene expression in Neurospora. Proceedings of the National Academy of Sciences of the United States of America *100*, 13597-13602.

Crosthwaite, S.K., Loros, J.J., and Dunlap, J.C. (1995). Light-induced resetting of a circadian clock is mediated by a rapid increase in frequency transcript. Cell *81*, 1003-1012.

Crosthwaite, S.K., Dunlap, J.C., and Loros, J.J. (1997). Neurospora wc-1 and wc-2: Transcription, photoresponses, and the origins of circadian rhythmicity. Science *276*, 763-769.

Cuadrado, A., and Nebreda, A. (2010). Mechanisms and functions of p38 MAPK signalling. Biochemical Journal *429*, 403-417.

Cuenda, A., and Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. Biochimica et Biophysica Acta *1773*, 1358-1375.

Daan, S., and Pittendrigh, C.S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. Journal of Comparative Physiology *106*, 253-266.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W.*, et al.* (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949-954.

Davis, R., DeSerres, D (1970). Genetic and microbiological research techniques for Neurospora crassa. Methods in Enzymology *27a*, 79-143.

Davis, S., Vanhoutte, P., Pagès, C., Caboche, J., and Laroche, S. (2000). The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. The Journal of Neuroscience *20*, 4563-4572.

de Paula, R.M., Lamb, T.M., Bennett, L., and Bell-Pedersen, D. (2008). A connection between MAPK pathways and circadian clocks. Cell Cycle *7*, 2630-2634.

DeCoursey, P.J., and Krulas, J.R. (1998). Behavior of SCN-lesioned chipmunks in natural habitat: A pilot study. Journal of Biological Rhythms *13*, 229-244.

Denault, D.L., Loros, J.J., and Dunlap, J.C. (2001). WC-2 mediates WC-1–FRQ interaction within the PAS protein-linked circadian feedback loop of Neurospora. The EMBO journal *20*, 109-117.

Dibner, C., Schibler, U., and Albrecht, U. (2010). The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. Annual Review of Physiology *72*, 517-549.

Dóczi, R., Brader, G., Pettkó-Szandtner, A., Rajh, I., Djamei, A., Pitzschke, A., Teige, M., and Hirt, H. (2007). The Arabidopsis mitogen-activated protein kinase kinase MKK3 is upstream of group C mitogen-activated protein kinases and participates in pathogen signaling. The Plant Cell Online *19*, 3266-3279.

Dodd, A.N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., Hibberd, J.M., Millar, A.J., and Webb, A.A.R. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science *309*, 630-633.

Doherty, C.J., and Kay, S.A. (2010). Circadian control of global gene expression patterns. Annual Review of Genetics *44*, 419-444.

Duffield, G.E., Best, J.D., Meurers, B.H., Bittner, A., Loros, J.J., and Dunlap, J.C. (2002). Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. Current Biology *12*, 551-557.

Dunlap, J., Loros, J., Colot, H., Mehra, A., Belden, W., Shi, M., Hong, C., Larrondo, L., Baker, C., and Chen, C. (2007). A circadian clock in Neurospora: How genes and proteins cooperate to produce a sustained, entrainable, and compensated biological oscillator with a period of about a day. Paper presented at: Cold Spring Harbor Symposia on Quantitative Biology.

Dunlap, J.C., Loros, J.J., Aronson, B.D., Merrow, M., Crosthwaite, S., Bell-Pedersen, D., Johnson, K., Lindgren, K., and Garceau, N.Y. (1995). The genetic basis of the circadian clock: Identification of frq and FRQ as clock components in Neurospora. Ciba Foundation Symposium *183*, 3-17; discussion 17-25.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. Cell *96*, 271-290.

Dunlap, J.C., Loros, J.J., Liu, Y., and Crosthwaite, S.K. (1999). Eukaryotic circadian systems: Cycles in common. Genes to Cells *4*, 1-10.

Dunlap, J.C., and Loros, J.J. (2004). The Neurospora circadian system. Journal of Biological Rhythms *19*, 414-424.

Dunlap, J.C., Loros, J.J., and DeCoursey, P.J. (2004). Chronobiology : biological timekeeping (Sunderland, Mass.: Sinauer Associates).

Dziema, H., and Obrietan, K. (2002). PACAP potentiates L-type calcium channel conductance in suprachiasmatic nucleus neurons by activating the MAPK pathway. Journal of Neurophysiology *88*, 1374-1386.

Dziema, H., Oatis, B., Butcher, G.Q., Yates, R., Hoyt, K.R., and Obrietan, K. (2003). The ERK/MAP kinase pathway couples light to immediate-early gene expression in the suprachiasmatic nucleus. European Journal of Neuroscience *17*, 1617-1627.

Eckel-Mahan, K.L., Phan, T., Han, S., Wang, H., Chan, G.C.K., Scheiner, Z.S., and Storm, D.R. (2008). Circadian oscillation of hippocampal MAPK activity and cAMP: Implications for memory persistence. Nature Neuroscience *11*, 1074-1082.

Eide, E.J., Woolf, M.F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E.L., Giovanni, A., and Virshup, D.M. (2005). Control of mammalian circadian rhythm by CKIε-regulated proteasome-mediated PER2 degradation. Molecular and Cellular Biology *25*, 2795-2807.

Fabian, M.A., Biggs, W.H., Treiber, D.K., Atteridge, C.E., Azimioara, M.D., Benedetti, M.G., Carter, T.A., Ciceri, P., Edeen, P.T., Floyd, M.*, et al.* (2005). A small molecule-kinase interaction map for clinical kinase inhibitors. Nature Biotechnology *23*, 329-336.

Farnell, Y.F., Shende, V.R., Neuendorff, N., Allen, G.C., and Earnest, D.J. (2011). Immortalized cell lines for real-time analysis of circadian pacemaker and peripheral oscillator properties. European Journal of Neuroscience *33*, 1533-1540.

Feldman, J.F., and Hoyle, M.N. (1973). Isolation of circadian clock mutants of Neurospora crassa. Genetics *75*, 605-613.

Ferreiro, I., Joaquin, M., Islam, A., Gomez-Lopez, G., Barragan, M., Lombardía, L., Domínguez, O., Pisano, D., Lopez-Bigas, N., and Nebreda, A. (2010). Whole genome analysis of p38 SAPK-mediated gene expression upon stress. BMC Genomics *11*, 144.

Froehlich, A.C., Liu, Y., Loros, J.J., and Dunlap, J.C. (2002). White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science *297*, 815-819.

Froehlich, A.C., Loros, J.J., and Dunlap, J.C. (2003a). Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. Proceedings of the National Academy of Sciences of the United States of America *100*, 5914-5919.

Froehlich, A.C., Pregueiro, A., Lee, K., Denault, D., Colot, H., Nowrousian, M., Loros, J.J., and Dunlap, J.C. (2003b). The molecular workings of the Neurospora biological clock. Novartis Foundation Symposium *253*, 184-198; discussion 102-189, 198-202, 281-184.

Fu, L., Pelicano, H., Liu, J., Huang, P., and Lee, C.C. (2002). The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell *111*, 41-50.

Fu, L., and Lee, C.C. (2003). The circadian clock: Pacemaker and tumour suppressor. Nature Reviews Cancer *3*, 350-361.

Fujimura, M., Ochiai, N., Oshima, M., Motoyama, T., Ichiishi, A., Usami, R., Horikoshi, K., and Yamaguchi, I. (2003). Putative homologs of SSK22 MAPKK kinase and PBS2 MAPK kinase of Saccharomyces cerevisiae encoded by os-4 and os-5 genes for osmotic sensitivity and fungicide resistance in Neurospora crassa. Bioscience, Biotechnology, and Biochemistry *67*, 186-191.

Gaddameedhi, S., Reardon, J.T., Ye, R., Ozturk, N., and Sancar, A. (2012). Effect of circadian clock mutations on DNA damage response in mammalian cells. Cell Cycle *11*, 3481-3491.

Gallego, M., and Virshup, D.M. (2007). Post-translational modifications regulate the ticking of the circadian clock. Nature Reviews Molecular Cell Biology *8*, 139-148.

Gamsby, J.J., Loros, J.J., and Dunlap, J.C. (2009). A phylogenetically conserved DNA damage response resets the circadian clock. Journal of Biological Rhythms *24*, 193-202.

Garceau, N.Y., Liu, Y., Loros, J.J., and Dunlap, J.C. (1997). Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell *89*, 469-476.

Gardner, G.F., and Feldman, J.F. (1980). The frq locus in Neurospora crassa: A key element in circadian clock organization. Genetics *96*, 877-886.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564-1569.

Gery, S., Komatsu, N., Baldjyan, L., Yu, A., Koo, D., and Koeffler, H.P. (2006). The circadian gene Per1 plays an important role in cell growth and DNA damage control in human cancer cells. Molecular Cell *22*, 375-382.

Gery, S., and Koeffler, H. (2007). The role of circadian regulation in cancer. Paper presented at: Cold Spring Harbor Symposia on Quantitative Biology (Cold Spring Harbor Laboratory Press).

Ginty, D.D., Kornhauser, J.M., Thompson, M.A., Bading, H., Mayo, K.E., Takahashi, J.S., and Greenberg, M.E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science *260*, 238-241.

Golden, S.S., Ishiura, M., Johnson, C.H., and Kondo, T. (1997). Cyanobacterial circadian rhythms. Annual Review of Plant Biology *48*, 327-354.

Gong, X., Liu, A., Ming, X., Deng, P., and Jiang, Y. (2010). UV-induced interaction between p38 MAPK and p53 serves as a molecular switch in determining cell fate. FEBS Letters *584*, 4711-4716.

Gooch, V.D., Mehra, A., Larrondo, L.F., Fox, J., Touroutoutoudis, M., Loros, J.J., and Dunlap, J.C. (2008). Fully codon-optimized luciferase uncovers novel temperature characteristics of the Neurospora clock. Eukaryotic Cell *7*, 28-37.

Grethe, S., Ares, M.P.S., Andersson, T., and Pörn-Ares, M.I. (2004). p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-xL. Experimental Cell Research *298*, 632-642.

Han, J., and Sun, P. (2007). The pathways to tumor suppression via route p38. Trends in Biochemical Sciences *32*, 364-371.

Hardin, P.E. (2005). The circadian timekeeping system of Drosophila. Current Biology *15*, R714-722.

Hasegawa, M., and Cahill, G.M. (2004). Regulation of the circadian oscillator in Xenopus retinal photoreceptors by protein kinases sensitive to the stress-activated protein kinase inhibitor, SB 203580. The Journal of Biological Chemistry *279*, 22738-22746.

Hayashi, Y., Sanada, K., and Fukada, Y. (2001). Circadian and photic regulation of MAP kinase by Ras- and protein phosphatase-dependent pathways in the chick pineal gland. FEBS Letters *491*, 71-75.

Hayashi, Y., Sanada, K., Hirota, T., Shimizu, F., and Fukada, Y. (2003). p38 mitogen-activated protein kinase regulates oscillation of chick pineal circadian clock. The Journal of Biological Chemistry *278*, 25166-25171.

He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K.H., and Liu, Y. (2002). White collar-1, a DNA binding transcription factor and a light sensor. Science *297*, 840-843.

He, Q., Cheng, P., Yang, Y., He, Q., Yu, H., and Liu, Y. (2003). FWD1-mediated degradation of FREQUENCY in Neurospora establishes a conserved mechanism for circadian clock regulation. The EMBO journal *22*, 4421-4430.

He, Q., and Liu, Y. (2005a). Degradation of the Neurospora circadian clock protein FREQUENCY through the ubiquitin-proteasome pathway. Biochemical Society Transactions *33*, 953-956.

He, Q., and Liu, Y. (2005b). Molecular mechanism of light responses in Neurospora: From light-induced transcription to photoadaptation. Genes & Development *19*, 2888-2899.

He, Q., Shu, H., Cheng, P., Chen, S., Wang, L., and Liu, Y. (2005). Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the Neurospora circadian negative feedback loop. Journal of Biological Chemistry *280*, 17526-17532.

He, Q., Cha, J., He, Q., Lee, H.-C., Yang, Y., and Liu, Y. (2006). CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop. Genes & Development *20*, 2552-2565.

Heintzen, C., Loros, J.J., and Dunlap, J.C. (2001). The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. Cell *104*, 453-464.

Hess, J., Angel, P., and Schorpp-Kistner, M. (2004). AP-1 subunits: Quarrel and harmony among siblings. Journal of Cell Science *117*, 5965-5973.

Hirota, T., Lewis, W.G., Liu, A.C., Lee, J.W., Schultz, P.G., and Kay, S.A. (2008). A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3β. Proceedings of the National Academy of Sciences of the United States of America *105*, 20746-20751.

Ho, A.K., Mackova, M., Price, L., and Chik, C.L. (2003). Diurnal variation in p42/44 mitogen-activated protein kinase in the rat pineal gland. Molecular and Cellular Endocrinology *208*, 23-30.

Hohmann, S. (2002a). Osmotic stress signaling and osmoadaptation in yeasts. Microbiology and Molecular Biology Reviews *66*, 300-372.

Hohmann, S. (2002b). Osmotic adaptation in yeast--control of the yeast osmolyte system. International Review of Cytology *215*, 149-187.

Honda, S., and Selker, E.U. (2009). Tools for fungal proteomics: Multifunctional neurospora vectors for gene replacement, protein expression and protein purification. Genetics *182*, 11-23.

Huang, C., Ma, W.-Y., Maxiner, A., Sun, Y., and Dong, Z. (1999). p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. Journal of Biological Chemistry *274*, 12229-12235.

Irmler, S., Rogniaux, H., Hess, D., and Pillonel, C. (2006). Induction of OS-2 phosphorylation in Neurospora crassa by treatment with phenylpyrrole fungicides and osmotic stress. Pesticide Biochemistry and Physiology *84*, 25-37.

Isojima, Y., Nakajima, M., Ukai, H., Fujishima, H., Yamada, R.G., Masumoto, K.-h., Kiuchi, R., Ishida, M., Ukai-Tadenuma, M., Minami, Y.*, et al.* (2009). CKIε/δ-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. Proceedings of the National Academy of Sciences of the United States of America *106*, 15744-15749.

Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., De Vries, G.J., and Reppert, S.M. (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell *96*, 57-68.

Jinlian, L., Yingbin, Z., and Chunbo, W. (2007). p38 MAPK in regulating cellular responses to ultraviolet radiation. Journal of Biomedical Science *14*, 303-312.

Johnson, C.H., Elliott, J.A., and Foster, R. (2003). Entrainment of circadian programs. Chronobiology International *20*, 741-774.

Johnson, G.L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298*, 1911-1912.

Johnson, L., Cao, X., and Jacobsen, S. (2002). Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Current Biology *12*, 1360-1367.

Jones, C.A., Greer-Phillips, S.E., and Borkovich, K.A. (2007). The response regulator RRG-1 functions upstream of a mitogen-activated protein kinase pathway impacting asexual development, female fertility, osmotic stress, and fungicide resistance in Neurospora crassa. Molecular Biology of the Cell *18*, 2123-2136.

Kang, T.-H., Reardon, J.T., and Sancar, A. (2011). Regulation of nucleotide excision repair activity by transcriptional and post-transcriptional control of the XPA protein. Nucleic Acids Research *39*, 3176-3187.

Karmarkar, S.W., Bottum, K.M., Krager, S.L., and Tischkau, S.A. (2011). ERK/MAPK is essential for endogenous neuroprotection in SCN2.2 cells. PLoS One *6*, e23493.

Keller, M., Mazuch, J., Abraham, U., Eom, G.D., Herzog, E.D., Volk, H.-D., Kramer, A., and Maier, B. (2009). A circadian clock in macrophages controls inflammatory immune responses. Proceedings of the National Academy of Sciences of the United States of America *106*, 21407-21412.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iε. Cell *94*, 97-107.

Ko, C.H., and Takahashi, J.S. (2006). Molecular components of the mammalian circadian clock. Human Molecular Genetics *15*, R271-R277.

Ko, G.Y.P., Ko, M.L., and Dryer, S.E. (2001). Circadian regulation of cGMP-gated cationic channels of chick retinal cones: Erk MAP kinase and Ca2+/calmodulin-dependent protein kinase II. Neuron *29*, 255-266.

Ko, M.L., Liu, Y., Dryer, S.E., and Ko, G.Y. (2007). The expression of L-type voltage-gated calcium channels in retinal photoreceptors is under circadian control. Journal of Neurochemistry *103*, 784-792.

Ko, M.L., Shi, L., and Ko, G.Y. (2009). Circadian controls outweigh acute illumination effects on the activity of extracellular signal-regulated kinase (ERK) in the retina. Neuroscience Letters *451*, 74-78.

Ko, M.L., Shi, L., Tsai, J.-Y., Young, M.E., Neuendorff, N., Earnest, D.J., and Ko, G.Y.-P. (2011). Cardiac-specific mutation of clock alters the quantitative measurements of physical activities without changing behavioral circadian rhythms. Journal of Biological Rhythms *26*, 412-422.

Koike, N., Yoo, S.-H., Huang, H.-C., Kumar, V., Lee, C., Kim, T.-K., and Takahashi, J.S. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science *338*, 349-354.

Kolch, W. (2005). Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nature Reviews Molecular Cell Biology *6*, 827-837.

Korotayev, K., Chaussepied, M., and Ginsberg, D. (2008). ERK activation is regulated by E2F1 and is essential for E2F1-induced S phase entry. Cellular Signalling *20*, 1221-1226.

Kozma-Bognár, L., and Kaldi, K. (2008). Synchronization of the fungal and the plant circadian clock by light. ChemBioChem *9*, 2565-2573.

Krantz, M., Becit, E., and Hohmann, S. (2006). Comparative genomics of the HOG-signalling system in fungi. Current Genetics *49*, 137-151.

Lamb, T.M., Goldsmith, C.S., Bennett, L., Finch, K.E., and Bell-Pedersen, D. (2011). Direct transcriptional control of a p38 MAPK pathway by the circadian clock in Neurospora crassa. PLoS One *6*, e27149.

Lamb, T.M., Finch, K.E., and Bell-Pedersen, D. (2012). The Neurospora crassa OS MAPK pathway-activated transcription factor ASL-1 contributes to circadian rhythms in pathway responsive clock-controlled genes. Fungal Genetics and Biology *49*, 180-188.

Lee, H.S., Nelms, J.L., Nguyen, M., Silver, R., and Lehman, M.N. (2003). The eye is necessary for a circadian rhythm in the suprachiasmatic nucleus. Nature Neuroscience *6*, 111-112.

Lee, J.-E., and Edery, I. (2008). Circadian regulation in the ability of Drosophila to combat pathogenic infections. Current Biology *18*, 195-199.

Lonze, B.E., and Ginty, D.D. (2002). Function and regulation of CREB family transcription factors in the nervous system. Neuron *35*, 605-623.

Loros, J.J. (1998). Time at the end of the millennium: The Neurospora clock. Current Opinion in Microbiology *1*, 698-706.

Loros, J.J., Dunlap, J.C., Larrondo, L.F., Shi, M., Belden, W.J., Gooch, V.D., Chen, C.H., Baker, C.L., Mehra, A., Colot, H.V*., et al.* (2007). Circadian output, input, and intracellular oscillators: Insights into the circadian systems of single cells. Cold Spring Harbor Symposia on Quantitative Biology *72*, 201-214.

Louie, K., and Wilson, M.A. (2001). Temporally structured replay of awake hippocampal ensemble activity during rapid eye movement sleep. Neuron *29*, 145-156.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science *288*, 483-491.

Luo, C., Loros, J.J., and Dunlap, J.C. (1998). Nuclear localization is required for function of the essential clock protein FRQ. The EMBO journal *17*, 1228-1235.

Luo, J., Phan, T.X., Yang, Y., Garelick, M.G., and Storm, D.R. (2013). Increases in cAMP, MAPK activity, and CREB phosphorylation during REM sleep: Implications for REM sleep and memory consolidation. The Journal of Neuroscience *33*, 6460-6468.

Menet, J.S., Rodriguez, J., Abruzzi, K.C., and Rosbash, M. (2012). Nascent-seq reveals novel features of mouse circadian transcriptional regulation. eLife *1*.

Meng, Q.-J., Maywood, E.S., Bechtold, D.A., Lu, W.-Q., Li, J., Gibbs, J.E., Dupré, S.M., Chesham, J.E., Rajamohan, F., Knafels, J*., et al.* (2010). Entrainment of disrupted circadian behavior through inhibition of casein kinase 1 (CK1) enzymes. Proceedings of the National Academy of Sciences of the United States of America *107*, 15240-15245.

Merrow, M., Franchi, L., Dragovic, Z., Gorl, M., Johnson, J., Brunner, M., Macino, G., and Roenneberg, T. (2001). Circadian regulation of the light input pathway in Neurospora crassa. The EMBO journal *20*, 307-315.

Merrow, M.W., Garceau, N.Y., and Dunlap, J.C. (1997). Dissection of a circadian oscillation into discrete domains. Proceedings of the National Academy of Sciences of the United States of America *94*, 3877-3882.

Mohawk, J.A., Green, C.B., and Takahashi, J.S. (2012). Central and peripheral circadian clocks in mammals. Annual Review of Neuroscience *35*, 445-462.

Morrison, D.K., and Davis, R.J. (2003). Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annual Review of Cell and Developmental Biology *19*, 91-118.

Muñoz, E., Brewer, M., and Baler, R. (2002). Circadian transcription thinking outside the E-box. Journal of Biological Chemistry *277*, 36009-36017.

Nakaya, M., Sanada, K., and Fukada, Y. (2003). Spatial and temporal regulation of mitogen-activated protein kinase phosphorylation in the mouse suprachiasmatic nucleus. Biochemical and Biophysical Research Communications *305*, 494-501.

Noguchi, R., Banno, S., Ichikawa, R., Fukumori, F., Ichiishi, A., Kimura, M., Yamaguchi, I., and Fujimura, M. (2007). Identification of OS-2 MAP kinase-dependent genes induced in response to osmotic stress, antifungal agent fludioxonil, and heat shock in Neurospora crassa. Fungal Genetics and Biology *44*, 208-218.

O'Rourke, S.M., and Herskowitz, I. (1998). The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in Saccharomyces cerevisiae. Genes & Development *12*, 2874-2886.

Obrietan, K., Impey, S., and Storm, D.R. (1998). Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nature Neuroscience *1*, 693-700.

Owens, D.M., and Keyse, S.M. (2007). Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene *26*, 3203-3213.

Panda, S., Hogenesch, J.B., and Kay, S.A. (2003). Circadian light input in plants, flies and mammals. Paper presented at: Molecular Clocks and Light Signalling: Novartis Foundation Symposium 253 (Wiley Online Library).

Petrzilka, S., Taraborrelli, C., Cavadini, G., Fontana, A., and Birchler, T. (2009). Clock gene modulation by TNF-alpha depends on calcium and p38 MAP kinase signaling. Journal of Biological Rhythms *24*, 283-294.

Phan, T.H., Chan, G.C.-K., Sindreu, C.B., Eckel-Mahan, K.L., and Storm, D.R. (2011). The diurnal oscillation of MAP (mitogen-activated protein) kinase and adenylyl cyclase activities in the hippocampus depends on the suprachiasmatic nucleus. The Journal of Neuroscience *31*, 10640-10647.

Pittenger, C., Huang, Y.Y., Paletzki, R.F., Bourtchouladze, R., Scanlin, H., Vronskaya, S., and Kandel, E.R. (2002). Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. Neuron *34*, 447-462.

Pizzio, G.A., Hainich, E.C., Ferreyra, G.A., Coso, O.A., and Golombek, D.A. (2003). Circadian and photic regulation of ERK, JNK and p38 in the hamster SCN. Neuroreport *14*, 1417-1419.

Plautz, J.D., Kaneko, M., Hall, J.C., and Kay, S.A. (1997). Independent photoreceptive circadian clocks throughout Drosophila. Science *278*, 1632-1635.

Poe, G.R., Nitz, D.A., McNaughton, B.L., and Barnes, C.A. (2000). Experience-dependent phase-reversal of hippocampal neuron firing during REM sleep. Brain Research *855*, 176-180.

Pollard, L.C., Choy, E.H., Gonzalez, J., Khoshaba, B., and Scott, D.L. (2006). Fatigue in rheumatoid arthritis reflects pain, not disease activity. Rheumatology *45*, 885-889.

Porras, A., Zuluaga, S., Black, E., Valladares, A., Alvarez, A.M., Ambrosino, C., Benito, M., and Nebreda, A.R. (2004). p38α mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. Molecular Biology of the Cell *15*, 922-933.

Posas, F., Chambers, J.R., Heyman, J.A., Hoeffler, J.P., de Nadal, E., and Ariño, J.n. (2000). The transcriptional response of yeast to saline stress. Journal of Biological Chemistry *275*, 17249-17255.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M.W. (1998). Double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. Cell *94*, 83-95.

Ramsey, K.M., Yoshino, J., Brace, C.S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H.-K., Chong, J.L., Buhr, E.D., Lee, C.*, et al.* (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. Science *324*, 651-654.

Ripperger, J.A., Shearman, L.P., Reppert, S.M., and Schibler, U. (2000). CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. Genes & Development *14*, 679-689.

Roenneberg, T., and Merrow, M. (1998). Molecular circadian oscillators: An alternative hypothesis. Journal of Biological Rhythms *13*, 167-179.

Rosato, E., and Kyriacou, C.P. (2001). Flies, clocks and evolution. Philosophical Transactions of the Royal Society B: Biological Sciences *356*, 1769-1778.

Roux, P.P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiology and Molecular Biology Reviews *68*, 320-344.

Sanada, K., Hayashi, Y., Harada, Y., Okano, T., and Fukada, Y. (2000). Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. The Journal of Neuroscience *20*, 986-991.

Sanada, K., Okano, T., and Fukada, Y. (2002). Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. The Journal of Biological Chemistry *277*, 267-271.

Sanada, K., Harada, Y., Sakai, M., Todo, T., and Fukada, Y. (2004). Serine phosphorylation of mCRY1 and mCRY2 by mitogen-activated protein kinase. Genes to Cells *9*, 697-708.

Sargent, M.L., Briggs, W.R., and Woodward, D.O. (1966). Circadian nature of a rhythm expressed by an invertaseless strain of Neurospora crassa. Journal of Plant Physiology *41*, 1343-1349.

Schafmeier, T., Haase, A., Kaldi, K., Scholz, J., Fuchs, M., and Brunner, M. (2005). Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell *122*, 235-246.

Schernhammer, E.S., Laden, F., Speizer, F.E., Willett, W.C., Hunter, D.J., Kawachi, I., and Colditz, G.A. (2001). Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. Journal of the National Cancer Institute *93*, 1563-1568.

Schibler, U. (2006). Circadian time keeping: The daily ups and downs of genes, cells, and organisms. Progress in Brain Research *153*, 271-282.

Schmidt, T.M., Chen, S.K., and Hattar, S. (2011). Intrinsically photosensitive retinal ganglion cells: Many subtypes, diverse functions. Trends in Neurosciences *34*, 572-580.

Schurov, I.L., Hepworth, T.J., and Hastings, M.H. (2002). Dopaminergic signalling in the rodent neonatal suprachiasmatic nucleus identifies a role for protein kinase A and mitogen-activated protein kinase in circadian entrainment. European Journal of Neuroscience *15*, 223-232.

Shimizu, K., Okada, M., Takano, A., and Nagai, K. (1999). SCOP, a novel gene product expressed in a circadian manner in rat suprachiasmatic nucleus. FEBS Letters *458*, 363-369.

Shimizu, K., Okada, M., Nagai, K., and Fukada, Y. (2003). Suprachiasmatic nucleus circadian oscillatory protein, a novel binding partner of K-Ras in the membrane rafts, negatively regulates MAPK pathway. Journal of Biological Chemistry *278*, 14920-14925.

Shimizu, K., Phan, T., Mansuy, I.M., and Storm, D.R. (2007). Proteolytic degradation of SCOP in the hippocampus contributes to activation of MAP kinase and memory. Cell *128*, 1219-1229.

Sindreu, C.B., Scheiner, Z.S., and Storm, D.R. (2007). Ca2+-stimulated adenylyl cyclases regulate ERK-dependent activation of MSK1 during fear conditioning. Neuron *53*, 79-89.

Smith, K.M., Sancar, G., Dekhang, R., Sullivan, C.M., Li, S., Tag, A.G., Sancar, C., Bredeweg, E.L., Priest, H.D., McCormick, R.F*., et al.* (2010). Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for Neurospora white collar complex. Eukaryotic Cell *9*, 1549-1556.

Spriggs, D.R., Sherman, M.L., Michie, H., Arthur, K.A., Imamura, K., Wilmore, D., Frei, E., and Kufe, D.W. (1988). Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion: A phase I and pharmacologic study. Journal of the National Cancer Institute *80*, 1039-1044.

Storch, K.-F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H., and Weitz, C.J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78-83.

Straub, R.H., and Cutolo, M. (2007). Circadian rhythms in rheumatoid arthritis: Implications for pathophysiology and therapeutic management. Arthritis & Rheumatism *56*, 399-408.

Talora, C., Franchi, L., Linden, H., Ballario, P., and Macino, G. (1999). Role of a white collar-1-white collar-2 complex in blue-light signal transduction. Science Signaling *18*, 4961.

Tauber, E., Last, K.S., Olive, P.J.W., and Kyriacou, C.P. (2004). Clock gene evolution and functional divergence. Journal of Biological Rhythms *19*, 445-458.

Tischkau, S.A., Mitchell, J.W., Tyan, S.-H., Buchanan, G.F., and Gillette, M.U. (2003). Ca2+/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. Journal of Biological Chemistry *278*, 718-723.

Tsuchiya, Y., Minami, I., Kadotani, H., Todo, T., and Nishida, E. (2013). Circadian clock-controlled diurnal oscillation of Ras/ERK signaling in mouse liver. Proceedings of the Japan Academy Series B, Physical and Biological Sciences *89*, 59-65.

Uchida, Y., Osaki, T., Yamasaki, T., Shimomura, T., Hata, S., Horikawa, K., Shibata, S., Todo, T., Hirayama, J., and Nishina, H. (2012). Involvement of stress kinase mitogen-activated protein kinase kinase 7 in regulation of mammalian circadian clock. Journal of Biological Chemistry *287*, 8318-8326.

Vanhoutte, P., Barnier, J.-V., Guibert, B., Pagès, C., Besson, M.-J., Hipskind, R.A., and Caboche, J. (1999). Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. Molecular and Cellular Biology *19*, 136-146.

Vanselow, K., Vanselow, J.T., Westermark, P.O., Reischl, S., Maier, B., Korte, T., Herrmann, A., Herzel, H., Schlosser, A., and Kramer, A. (2006). Differential effects of PER2 phosphorylation: Molecular basis for the human familial advanced sleep phase syndrome (FASPS). Genes & Development *20*, 2660-2672.

Vgontzas, A.N., Zoumakis, E., Lin, H.-M., Bixler, E.O., Trakada, G., and Chrousos, G.P. (2004). Marked decrease in sleepiness in patients with sleep apnea by etanercept, a tumor necrosis factor-α antagonist. Journal of Clinical Endocrinology & Metabolism *89*, 4409-4413.

Vitalini, M.W., de Paula, R.M., Park, W.D., and Bell-Pedersen, D. (2006). The rhythms of life: Circadian output pathways in Neurospora. Journal of Biological Rhythms *21*, 432-444.

Vitalini, M.W., de Paula, R.M., Goldsmith, C.S., Jones, C.A., Borkovich, K.A., and Bell-Pedersen, D. (2007). Circadian rhythmicity mediated by temporal regulation of the activity of p38 MAPK. Proceedings of the National Academy of Sciences of the United States of America *104*, 18223-18228.

Wagner, E.F., and Nebreda, A.R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nature Reviews Cancer *9*, 537-549.

Wang, G.K., and Sehgal, A. (2002). Signaling components that drive circadian rhythms. Current Opinion in Neurobiology *12*, 331-338.

Waskiewicz, A.J., and Cooper, J.A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. Current Opinion in Cell Biology *7*, 798-805.

Watanabe, S., Yamashita, K., Ochiai, N., Fukumori, F., Ichiishi, A., Kimura, M., and Fujimura, M. (2007). OS-2 mitogen activated protein kinase regulates the clock-controlled gene ccg-1 in Neurospora crassa. Bioscience, Biotechnology, and Biochemistry *71*, 2856-2859.

Weber, F., Hung, H.C., Maurer, C., and Kay, S.A. (2006). Second messenger and Ras/MAPK signalling pathways regulate CLOCK/CYCLE-dependent transcription. Journal of Neurochemistry *98*, 248-257.

Whitmarsh, A.J., and Davis, R.J. (1998). Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends in Biochemical Sciences *23*, 481-485.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiological Reviews *79*, 143-180.

Williams, J.A., Su, H.S., Bernards, A., Field, J., and Sehgal, A. (2001). A circadian output in Drosophila mediated by neurofibromatosis-1 and Ras/MAPK. Science *293*, 2251-2256.

Woelfle, M.A., Ouyang, Y., Phanvijhitsiri, K., and Johnson, C.H. (2004). The adaptive value of circadian clocks: An experimental assessment in cyanobacteria. Current Biology *14*, 1481-1486.

Wu, Z.L., Thomas, S.A., Villacres, E.C., Xia, Z., Simmons, M.L., Chavkin, C., Palmiter, R.D., and Storm, D.R. (1995). Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. Proceedings of the National Academy of Sciences of the United States of America *92*, 220-224.

Xu, Y., Toh, K.L., Jones, C.R., Shin, J.Y., Fu, Y.H., and Ptáček, L.J. (2007). Modeling of a human circadian mutation yields insights into clock regulation by PER2. Cell *128*, 59-70.

Yadav, G., Straume, M., Heath, J., 3rd, and Zatz, M. (2003). Are changes in MAPK/ERK necessary or sufficient for entrainment in chick pineal cells? The Journal of Neuroscience *23*, 10021-10031.

Yagita, K., Yamanaka, I., Koinuma, S., Shigeyoshi, Y., and Uchiyama, Y. (2009). Mini screening of kinase inhibitors affecting period-length of mammalian cellular circadian clock. Acta Histochemica et Cytochemica *42*, 89-93.

Yang, Y., Cheng, P., Zhi, G., and Liu, Y. (2001). Identification of a calcium/calmodulin-dependent protein kinase that phosphorylates the Neurospora circadian clock protein FREQUENCY. Journal of Biological Chemistry *276*, 41064-41072.

Yang, Y., Cheng, P., and Liu, Y. (2002). Regulation of the Neurospora circadian clock by casein kinase II. Genes & Development *16*, 994-1006.

Yang, Y., Cheng, P., He, Q., Wang, L., and Liu, Y. (2003). Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the Neurospora circadian clock. Molecular and Cellular Biology *23*, 6221-6228.

Yang, Y., He, Q., Cheng, P., Wrage, P., Yarden, O., and Liu, Y. (2004). Distinct roles for PP1 and PP2A in the Neurospora circadian clock. Genes & Development *18*, 255-260.

Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W., Mischak, H.*, et al.* (1999). Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature *401*, 173-177.

Yoo, S.-H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.-K., Oh, W.J., Yoo, O.J.*, et al.* (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proceedings of the National Academy of Sciences of the United States of America *101*, 5339-5346.

Yoo, S.-H., Ko, C.H., Lowrey, P.L., Buhr, E.D., Song, E.-j., Chang, S., Yoo, O.J., Yamazaki, S., Lee, C., and Takahashi, J.S. (2005). A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proceedings of the National Academy of Sciences of the United States of America *102*, 2608-2613.

Yoshitane, H., Honma, S., Imamura, K., Nakajima, H., Nishide, S.-y., Ono, D., Kiyota, H., Shinozaki, N., Matsuki, H., Wada, N.*, et al.* (2012). JNK regulates the photic response of the mammalian circadian clock. EMBO Reports *13*, 455-461.

Young, M.W., and Kay, S.A. (2001). Time zones: A comparative genetics of circadian clocks. Nature Reviews Genetics *2*, 702-715.

Zarubin, T., and Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. Cell Research *15*, 11-18.

Zhang, Y., Lamm, R., Pillonel, C., Lam, S., and Xu, J.-R. (2002). Osmoregulation and fungicide resistance: The Neurospora crassa os-2 gene encodes a HOG1 mitogen-activated protein kinase homologue. Applied and Environmental Microbiology *68*, 532-538.

APPENDIX A

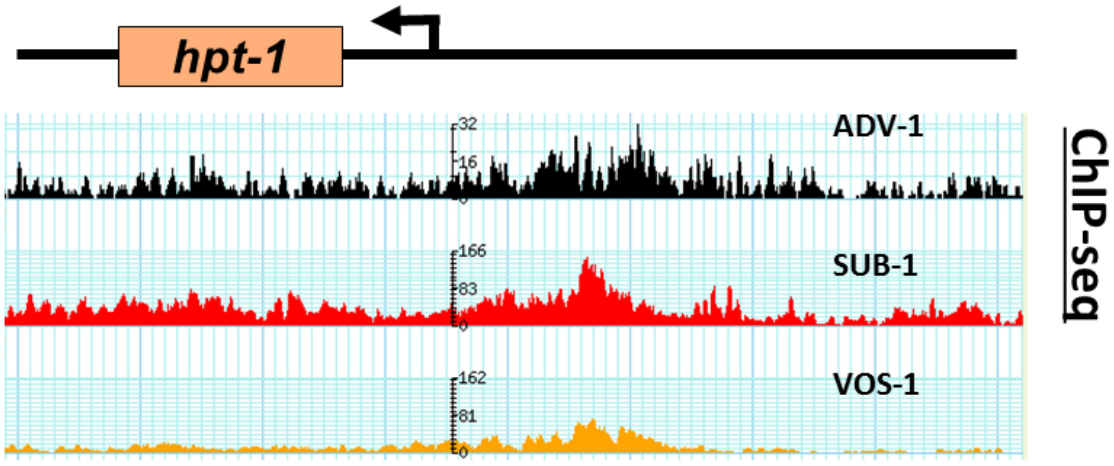


Figure A-1. First-tier transcription factors bind the promoter of *hpt-1*. After a light pulse, ChIP was performed on first-tier transcription factors that were targets of the WCC. DNA that was immunoprecipitated in complex with the transcription factors was analyzed by Illumina sequencing. The tracts of data are histograms displaying the number of sequence reads that were purified through ChIP of each transcription factor along the genomic locus of *hpt-1*.

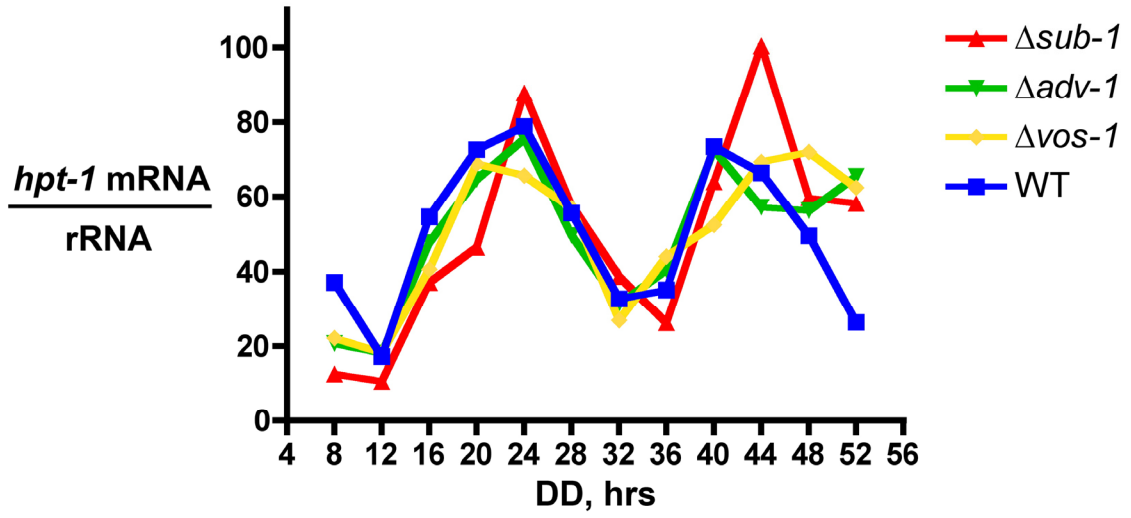


Figure A-2. ***hpt-1*** **mRNA accumulation is rhythmic in transcription factor knockout mutants.** Wild type and transcription factor knockout strains of Neurospora were grown in liquid culture in DD and collected every 4 hrs. mRNA levels were determined by northern blotting, quantified by densitometry, an normalized to rRNA levels. Each trace represents normalized mRNA values for each indicated strain (n=1). The average of values across timepoints was to 50 for each strain. Data were derived from one experiment in which all strains were grown and analyzed simultaneously.

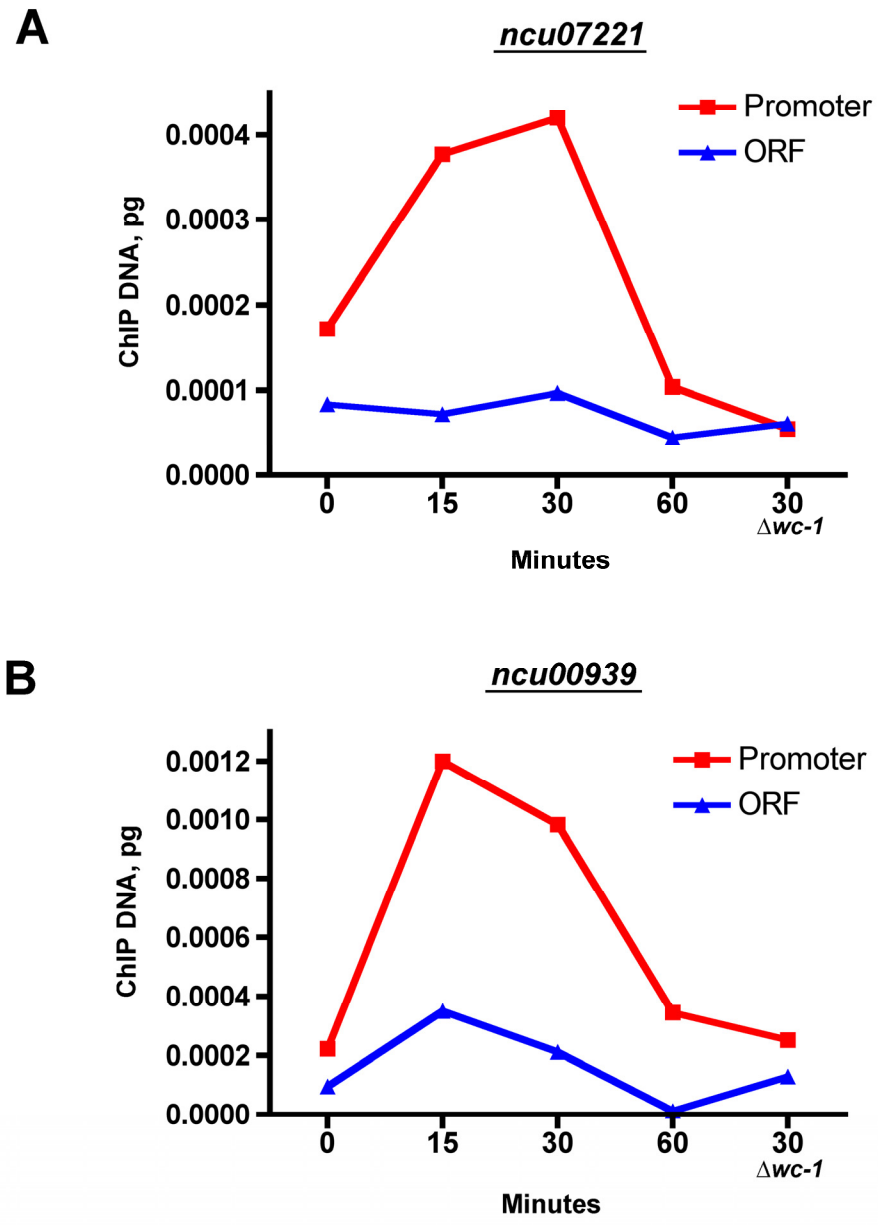


Figure A-3. The promoters of histidine kinase genes *ncu07221* and *ncu00939* are bound by the WCC in response to a light pulse. Graph represents amount of WC-2 ChIP DNA obtained from either the promoter or open reading frame (ORF) of *ncu07221* (A) or *ncu00939* (B) (n=1). Cultures were grown in DD for 24 hrs and then exposed to a light pulse for the time indicated. In each culture, chromatin immunoprecipitation with an anti-WC-2 antibody was used to purify genomic DNA bound by the WCC. ChIP DNA was analyzed using qPCR with primers that amplified either the promoter of the gene (red squares) or the ORF (blue triangles), and DNA was quantified by the absolute quantification method.

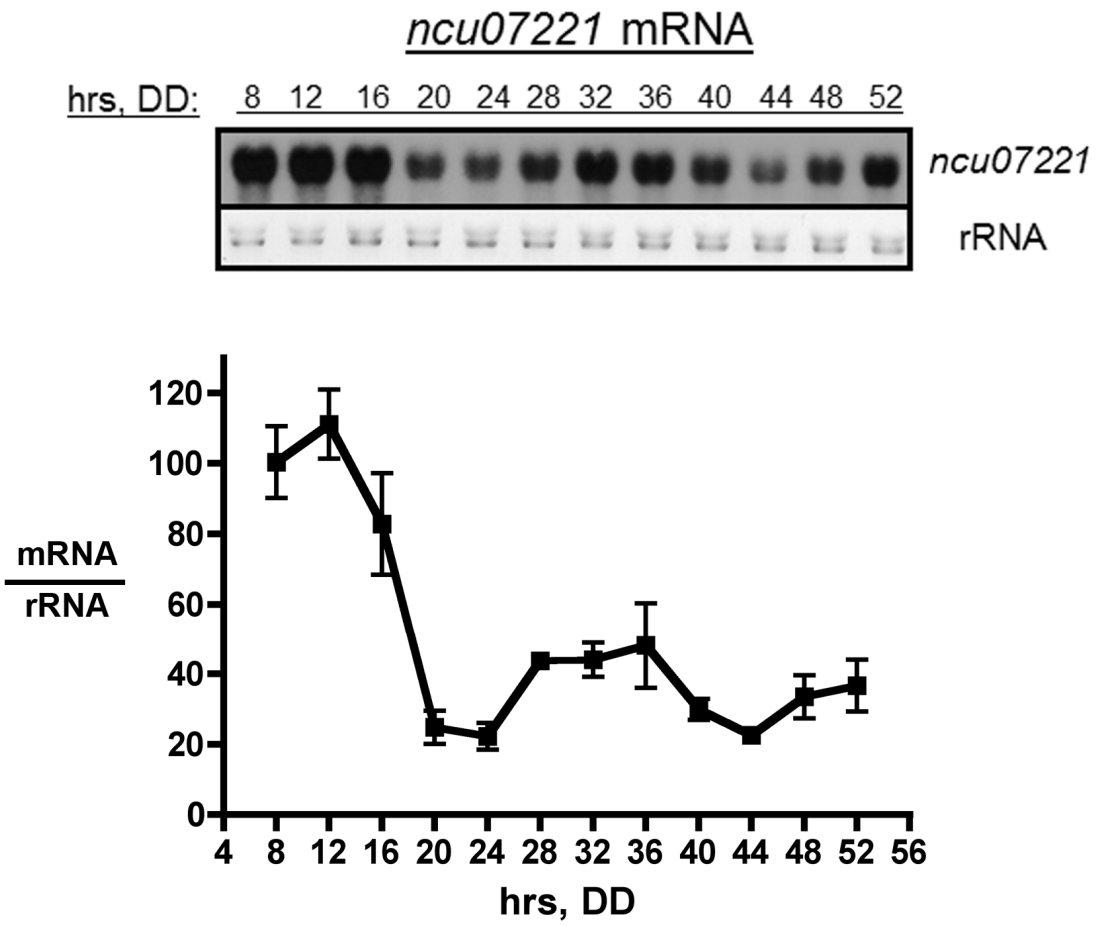


Figure A-4. The genes *ncu07221* is expressed with a circadian rhythm. Representative northern blot showing *ncu07221* mRNA levels from wild type cultures grown in DD and collected at 4 h intervals. rRNA is used as the loading control. Densitometric analysis of northern blot experiments are shown in the graph (n=5, ±SEM). The average value across timepoints was set to 50.